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**Development and validation of capillary electrophoresis assays for the
determination of the stereochemical purity of drug substances**

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ABBREVIATIONS

α -CD	α -cyclodextrin
β -CD	β -cyclodextrin
γ -CD	γ -cyclodextrin
BGE	Background electrolyte
BP	British Pharmacopoeia
CD(s)	Cyclodextrin(s)
CD-modified MEEKC	Cyclodextrin-modified microemulsion electrokinetic chromatography
CE	Capillary electrophoresis
Carboxymethyl α -CD	Carboxymethyl α -cyclodextrin
Carboxymethyl β -CD	Carboxymethyl β -cyclodextrin
CSPs	Chiral stationary phases
CZE	Capillary zone electrophoresis
DS	Degree of substitution
EOF	Electroosmotic flow
EMA	European Medicines Agency
FDA	Food and Drug Administration
HADS- β -CD	Heptakis-(2,3-di-O-acetyl-6-O-sulfo)- β -cyclodextrin
HPLC	High-performance liquid chromatography
ICH	International Conference on Harmonization
id	Internal diameter
LOD	Limit of detection
LOQ	Limit of quantitation
ME(s)	Microemulsion(s)
MEEKC	Microemulsion electrokinetic chromatography
MEKC	Micellar electrokinetic chromatography
NMR	Nuclear magnetic resonance
od	Outer diameter
Ph. Eur.	European Pharmacopoeia
PSPs	Pseudostationary phases
RSD	Relative standard deviation
SBE- β -CD	Sulfobutylether β -cyclodextrin
SDS	Sodium dodecyl sulfate
Sulfated α -CD	Sulfated α -cyclodextrin
Sulfated β -CD	Sulfated β -cyclodextrin
Sulfated γ -CD	Sulfated γ -cyclodextrin
Sulfopropyl α -CD	Sulfopropyl α -cyclodextrin
Sulfopropyl β -CD	Sulfopropyl β -cyclodextrin
USP	United States Pharmacopeia

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CHAPTER I

INTRODUCTION

1.1 Enantiomer and its importance

1.1.1 Definitions and overview of isomers ^[1,2,3]

Isomers are compounds that have the same stoichiometric molecular formula but differ in connectivity or spatial arrangement of their atoms. Isomers can be divided into two types; constitutional or structural isomers and stereoisomers. Constitutional isomers have a different connectivity or binding pattern of the atoms in three-dimensional space. For example, 1-propanol and 2-propanol possess the same molecular formula $C_4H_{10}O$, but their binding patterns are different (see Fig. 1). Stereoisomers have the same connectivity, but differ in the spatial orientation of some or all atoms in the three-dimensional space. Stereoisomers that their structures can be made identical by rotation around one or more single bonds are referred to conformational isomers. Conformational isomers are not included in this thesis. Only Configurational isomers will be described. Therefore, stereoisomers may be subdivided into two groups, i.e. enantiomers and diastereomers.

Enantiomers are nonsuperimposable mirror images of each other. For example, *R*-2-bromobutane and *S*-2-bromobutane have the same connectivity of their atoms, but the spatial arrangement of their atoms in the space is different (Fig. 1). The enantiomers of 2-bromobutane are mirror images but they cannot be superimposed. Nonsuperimposable mirror image is normally present in molecules bearing an asymmetric carbon atom or a chiral atom. Chiral atom has tetrahedral structures that are bonded by four different atoms or groups. In addition to the carbon atom, chiral centers can also be found in other elements such as silicon, nitrogen, phosphorus or sulfur. Compounds that their molecules are nonsuperimposable mirror image will be optically active. A pair of enantiomer can rotate the plane of plane-polarized light in equal amount or degree of rotation but in the opposite direction, so that enantiomers can also be classified as optical isomers (see Fig. 1). A pair of enantiomers has essentially identical physical and chemical properties in achiral media.

Diastereomers are stereoisomers that are not enantiomers. Thus, diastereomers are not related as mirror image of each other. Moreover, diastereomers may not always optically active. Unlike enantiomers, diastereomers generally have different physiochemical properties. Molecules having two or more stereogenic centers and geometric isomers that their structures are not related as mirror images are also considered to be diastereomers. The molecules bearing two or more stereogenic centers may have identical configuration at one or more stereogenic centers but opposite configuration at others. Geometric isomers possess molecules that have a restricted rotation at normal room temperatures and pressures. Restricted rotation in the molecules can be caused either by a double bond or a cyclic structure such as *cis*-1,2-dichloroethylene and *trans*-1,2-dichloroethylene bearing a double bond in the molecules (see also Fig. 1).

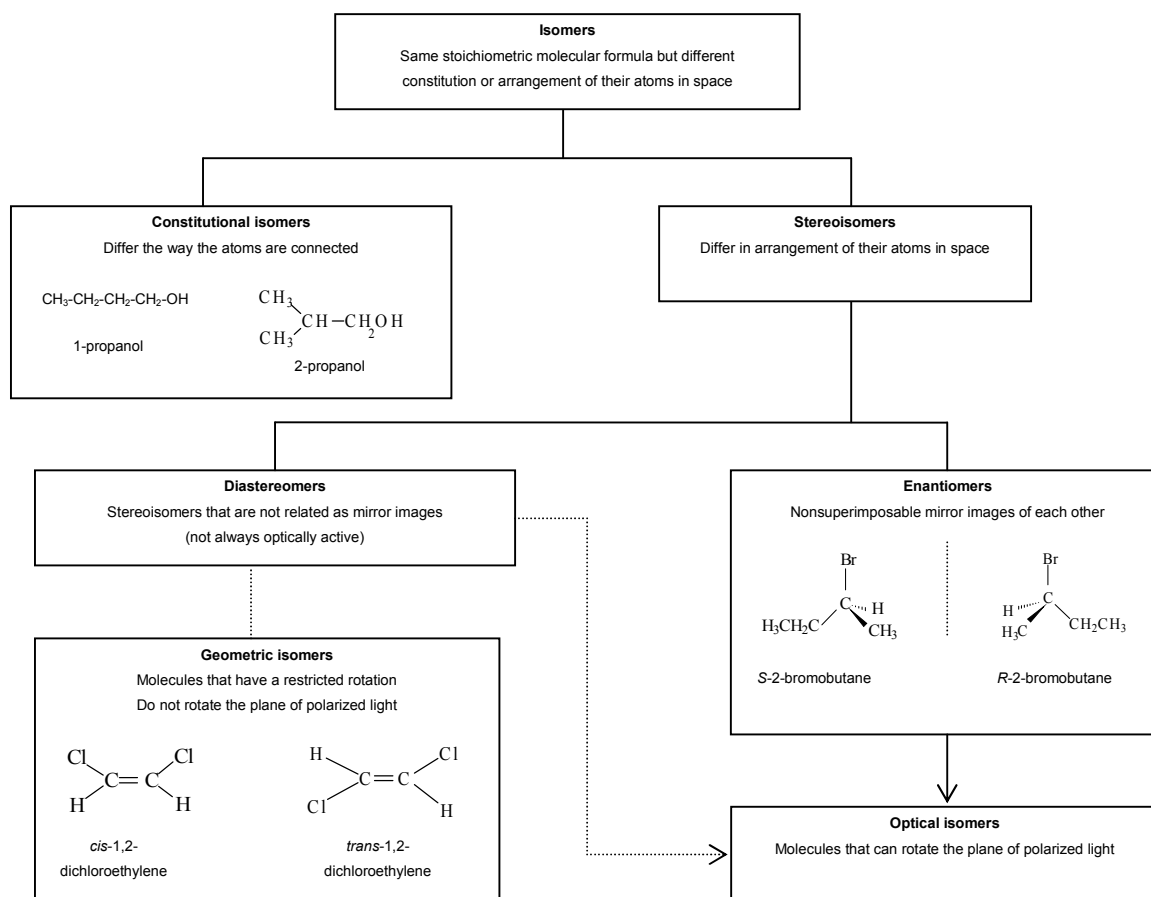


Figure 1. Overview of isomerism. (adapted from Ref. [1])

1.1.2 Enantiomers and biological activities

The stereoisomers play an important role in biological activity because of the stereospecificity for active and receptor sites. The interactions between the biologically active compounds and receptor proteins or enzymes often show a high or complete stereoselectivity. On the other hand, each isomer may have a different affinity to enzymes and the active sites of the receptors resulting in a different characteristic biological activity or toxicity. For example, while one enantiomer is active the other one enantiomer may have [2];

- *no activity*, for example, *S*-(+)-ibuprofen is an anti-inflammatory drug, the *R*-(-)-enantiomer has no activity,
- *the same type and strength of the activity*, for example, both the same isomers of the H blocker promazine have the same activity,
- *the same type of the activity but weaker potency*, for example, the *R*-(+)-enantiomer of the anticoagulant warfarin is less potent than the *S*-(-)-enantiomer,
- *a different activity*, for example, the *S*-(+)-enantiomer of ketamine is responsible for most of its anesthetic action while the *R*-(-)-enantiomer is responsible for its psychotic effects,
- *a toxicity*, for example, *S*-(-)-penicillamine is used as antiarthritic drug while *R*-(+)-penicillamine is extremely toxic.

Further examples of enantiomeric drugs that have different pharmacological and/or toxicological properties are shown in Table 1.

Table 1. The Different physiological properties of the enantiomers of some drugs [4]

Drugs	(+)-Enantiomer	(-)-Enantiomer
Barbiturates	Excitation	Sedation
Dobutamine	β_1 - and β_2 -adrenoceptor agonist (vasodilatation)	α_1 -Adrenoceptor agonist (positive inotropic / vasoconstriction)
Fluoxetine	Selective serotonin reuptake inhibitor	Minimal effect
Ketamine	Strong anesthetic	Weak anesthetic
Fenfluramine	Selective serotonin reuptake inhibitor	Norepinephrine / dopamine reuptake inhibitor (adverse side effect)
Levodopa	Antiparkinson	Agranulocytosis
Methadone	Minimal effect	Strong analgesic
Metamphetamine	Central nervous system stimulant	Peripheral vasodilator
Penicillamine	Antirheumatic (Wilson's disease)	Neurotoxic
Pentazocine	Antianxiety	Analgesic, respiratory depression
Propoxyphene	Analgesia	Antitussive
β -Adrenergic antagonist (e.g., propranolol)	Suppress ventricular arrhythmia without β -adrenergic blockade	Active β -adrenergic blocker
Morphine	Minimal effect	Strong analgesic
Thyroxine	Inactive	Thyroxemic effect
Verapamil	Minimal effect	Negative dromotropic; negative inotropic and chronotropic effects
Warfarin	Weak anticoagulant	Anticoagulant
Thalidomide	Sedative	Sedative, teratogenic
Picnadol	μ -Receptor agonist (analgesic)	Weak μ -receptor antagonist
Tetramisole	Minimal effect	Anthelmintic
Nonsteroidal anti-Inflammatory agents (NSAIDs)	Anti-inflammatory	Minimal effect

1.1.3 Enantiomers and drug development

In Table 1, the differences in drug action and/or toxicity of a pair of enantiomer are shown. One of the most recognized examples of an undesirable effect due to the use of a racemic drug is thalidomide. Thalidomide was synthesized in 1954 by Grünenthal Chemie in Germany under the brand name of Countergran and was subsequently licensed in other 46 countries [5]. The drug was first marketed in Germany in 1957 and was used as a sedative drug as well as an effective

antiemetic in pregnancy [5, 6]. In 1961 thalidomide was withdrawn from the market due to the fact that thalidomide caused teratogenic effects in pregnant women. Even though the cause of teratogenic effect is yet unclear, some experiments in animals suggested that the teratogenic effect may be caused by (S)-(-)-thalidomide [7–10]. The tragedy of thalidomide resulted in an awareness of using the racemic drugs and a stricter control as well as a reconsideration of the guidelines for new drug approval.

A new marketing strategy called racemic switch was launched due to the debates over racemic compounds and single enantiomers. The racemic switch offers the opportunity to the industrial companies to apply the single enantiomer compounds as new drugs even though their racemates have already been approved. Therefore many pharmaceutical companies have started to investigate the pharmacology and toxicology of individual enantiomers, and launch single pure enantiomer drugs to the market. An increase in the number of the single enantiomer drugs that were marketed from 2000 – 2005 is shown in Table 2. Moreover, Fig. 2 shows the proportions of achiral drugs, racemic drugs and single enantiomer drugs from year 1988 – 2002 highlighting an increase in the number of single enantiomer drugs in Japan.

Table 2. Worldwide sales of single enantiomer pharmaceutical products [11]

Therapeutic category	2000 sales (in \$ billions)	2004 sales (in \$ billions)	2005 sales (in \$ billions)	CAGR (%) [*] 2000-2005
Cardiovascular	27,650	34,033	36,196	6
Antibiotics and antifungals	25,942	32,305	34,298	6
Cancer therapies	12,201	21,358	27,172	17
Hematology	11,989	20,119	22,439	13
Hormone and endocrinology	15,228	20,608	22,355	8
Central nervous system	9,322	17,106	18,551	15
Respiratory	6,506	12,827	14,708	18
Antiviral	5,890	11,654	14,683	20
Gastrointestinal	4,171	11,647	13,476	26
Ophthalmic	2,265	3,063	3,416	9
Dermatological	1,272	1,486	1,561	4
Vaccines	1,427	2,450	3,100	17
Other	7,128	10,400	13,268	13
Total	130,991	199,056	225,223	11

^{*}CAGR is compound annual growth rate

Source: Technology Catalysts International

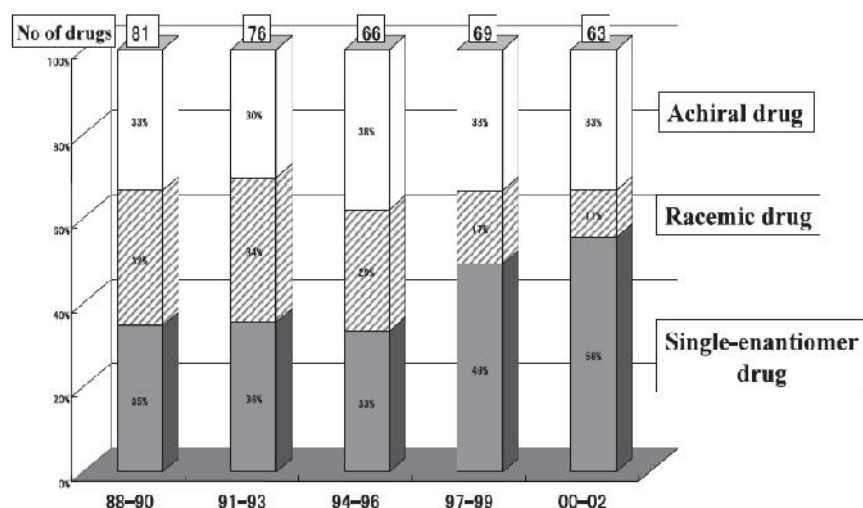


Figure 2. Number of Drug Approved in Japan during 1988 – 2002 [12]

1.2 Enantiomeric purity and regulatory agencies

As mentioned above single enantiomer drugs have become an important part of the overall pharmaceutical market. Generally, the safety and efficacy of the drug depend not only on the pharmacological and toxicological data of the active ingredient itself but also on the impurities. Therefore, the purity of stereoisomeric drug substances and products is an important issue. In the United States, the Food and Drug Administration (FDA) has stated a policy for new drug applications which requires appropriate information on the chemistry as well as the manufacturing and control data, such as methods and specifications, results of stability tests, proper labeling, pharmacological activity, pharmacokinetic profile, toxicology, and impurity limits [13]. For single enantiomers, pharmacology and toxicology evaluations must be conducted relying on the existing knowledge of the racemate. The European Medicines Agency (EMA) has stated guidelines on the quality, safety, and efficacy of medicinal products for human use [14] which are similar to the FDA guidelines. Besides the marketing considerations, the availability of single pure enantiomers is of importance in order to facilitate the studies of pharmacokinetics, pharmacodynamics, pharmacological and toxicological properties of single stereoisomeric compounds compared to racemic drugs that will ensure the safety and efficacy of the drugs.

The nature and quantity of impurities in a drug substance depend on different factors including the synthetic route, reaction conditions, quality of the starting material, reagents, solvents, purification steps, excipients, drug product manufacturing processes, packaging, and storage of the end product [15]. As a result by-products, intermediates and degradation products that are defined as pharmaceutical impurities can be found in drug substances and products. A pharmaceutical impurity is defined as any component of the drug substance or drug product that is not the active pharmaceutical ingredient. Impurity profiling which is a description of the identity as well as the quantity of impurities present in a drug substance or drug product is an important concern of the regulatory authorities. Thus, impurity profiling includes analytical activities with the aim of the detection, identification/structure elucidation and quantitative determination of organic and inorganic impurities as well as residual solvents in bulk drugs and pharmaceutical formulations [16].

In general, impurity limits (both specified and non-specified impurities) are listed in the monographs of pharmacopoeias, for example, the British Pharmacopoeia (BP), the European Pharmacopoeia, the Japanese Pharmacopoeia and the United State Pharmacopoeia (USP). The limits for related impurities vary from drug to drug and monograph to monograph. In the case of “classical” drugs usually 1.0-1.5% of total impurities and 0.5% of individual impurities are accepted. But in the case of new monographs the tendency is to limit total impurities down to about 0.5%. Furthermore, the International Conference on Harmonization (ICH) issued the regulations of impurities in new drug substances (ICH Q3A) [17] and drug products (ICH Q3B) [18]. The impurities can be classified into three groups based on ICH Q3A; organic impurities (process- and drug-related), inorganic impurities, and residual solvents. All potential impurities present in the new drug substances at a level greater than the reporting threshold (see Table 3) should be reported. Moreover, the structures of impurities should be identified if the impurities present at a level greater than the identification threshold. Identification of impurities present at an apparent level of not more than the identification threshold is generally not considered necessary, except for impurities that are expected to be unusually potent, producing toxic or pharmacological effects. Furthermore, if impurities present at a level greater than the qualification threshold, the impurities should be qualified for the biological safety. However, lower qualification thresholds can be appropriate if the impurities are unusually toxic.

Table 3. Drug substance impurities thresholds [17]

Maximum Daily Dose ¹	Reporting Threshold ^{2,3}	Identification Threshold ³	Qualification Threshold ³
≤ 2g/day	0.05%	0.10% or 1.0 mg per day intake (whichever is lower)	0.15% or 1.0 mg per day intake (whichever is lower)
> 2g/day	0.03%	0.05%	0.05%

¹ The amount of drug substance administered per day

² Higher reporting thresholds should be scientifically justified

³ Lower thresholds can be appropriate if the impurity is unusually toxic

In addition, specific tests and acceptance criteria have been addressed in the ICH guideline Q6A [19]. Regarding the ICH guideline Q6A on chiral drug substances which are developed as a single enantiomer, control of the other enantiomer should be considered in the same manner as for other impurities. Wherever the specified impurities, impurity limits and acceptance criteria listed in the monographs of pharmacopoeias are appropriate, they have to be utilized.

1.3 Separation and determination of enantiomeric impurities in drug substances

Since pairs of various enantiomers have been shown to have different therapeutic and toxicological properties, the identification and quantification of stereochemical impurities in raw materials and/or drug substances are an important part of drug development and regulatory assessment. The ICH guideline Q3A [17] recommended that the registration application for new drug substances should include documented evidence that the analytical procedures are validated and suitable for the

detection and quantification of impurities. The separation and determination of an enantiomeric impurity in drug substances can be accomplished by the “traditional” method polarimetry and modern methods including chiral high-performance liquid chromatography (HPLC) and chiral capillary electrophoresis (CE).

Traditional methods including the use of optical rotation often proved to be inadequate for the determination of enantiomeric impurities. However, polarimetry is still the simplest and most universal technique available to determine enantiomeric purity [20]. The main disadvantage of polarimetric method is that it requires knowledge of the specification of an optically pure sample and relatively large amounts of the substance are required for the quantification. Moreover, some chiral substances yield a small optical rotation and in such a situation the sensitivity of the polarimetric method is low. Therefore, the change in optical rotation as a function of concentration is small and can lead to a mistake in quantification.

Among chromatographic methods chiral HPLC is the most widely used as the analysis for enantioseparation because of a sufficient sensitivity, a wide variety of commercial columns, and of extensive application literatures [21]. The separation in HPLC can be performed by indirect separation using chiral derivatization reagents or by direct separation employing chiral stationary phases or chiral mobile phase additives. The indirect separation is based on the use of chiral derivatization reagents to form diastereomeric derivatives which differ in their chemical and physical properties and therefore can be separated on achiral stationary phases. The indirect separation is an alternative to an expensive chiral column and to compounds without a chromophore. It is more flexible, but the derivatization represents an additional step which can involve undesirable side reactions, the formation of decomposition products and racemization of chiral-derivatizing reagents [22, 23]. Moreover, the chiral derivatization reagents have to be of high enantiomeric purity and the presence of derivatizable groups in the analyte is a prerequisite. The direct approach using columns with chiral stationary phase is more convenient and also applicable for separations on a preparative scale. However, a collection of expensive columns to solve a variety of problems is required. The chiral mobile phase additive approach is a simple and flexible alternative which is not always applicable. The main disadvantage of chiral mobile phase additive is the high UV background noise if a chiral selector is UV absorbing. Moreover, the retention of the analyte may be difficult to predict and since the mobile phase contains the chiral selector it cannot be reused. Furthermore, relatively large amounts of the chiral selector are required.

Over the last two decades capillary electrophoresis (CE) has been acknowledged as a powerful analytical technique for chiral separations. It has become an alternative method to the chromatography, especially chiral HPLC, due to its simplicity, reliability, versatility, and low consumption of sample, chiral selector and reagents [24–29]. Moreover, CE has been employed as a suitable analytical method for drug-related impurity determinations in the routine laboratory [30]. The differences between HPLC and CE used as chiral separation techniques are compared and described in Table 5 which shows many advantages of using CE over HPLC. In recent years many fully validated CE methods have been described and CE is becoming a well established technique in academia as well as in the pharmaceutical industry [31]. Overall, CE with variety modes has

become one of advanced analytical methods for drug analysis in pharmaceutical, therapeutic, diagnostic as well as forensic applications as documented in many reviews [29, 32–37].

Table 5. Differences between HPLC and CE as chiral separation techniques [38]

	HPLC	CE
Instrument, cost, and safety	Expensive columns, consumption of a relatively high amount of buffer solutions, and hazardous organic modifiers.	Simple instrument: no pump, injector valves, and detector cells are required; a minute amount of solvent and extremely low amount of chiral selector and buffer are required; environmentally friendly and inexpensive.
Chiral selector	Immobilized; great number of commercially available CSPs; a combination of chiral selectors is difficult or at least time-consuming.	Commonly mobile, commercially available chiral selector are inexpensive, chiral selectors can be mixed in any desired ratio (only limited by solubility).
Selectivity and efficiency	Chiral separation selectivity may in the best case approach the thermodynamic selectivity of the chiral recognition but will never exceed it; the separation efficiency sometimes is poor.	Separation selectivity may easily exceed the thermodynamic selectivity of the recognition; a chiral separation even in the absence of chiral recognition is, in principle, feasible; high peak efficiency.
Manipulation of the mobility terms	Impossible to adjust the selectivity of chiral separation without changing the affinity pattern of the enantiomers toward chiral selector.	Possible to adjust the enantiomer migration order without reverting the affinity pattern between enantiomers of the analyte and a chiral selector.
Separation scale	Semi-preparative and preparative scale.	Analytical scale, very small sample volumes.
Method development	Relatively slow; changing and conditioning a column is time-consuming.	Rapid; changing a capillary and/ or chiral selector takes only few minutes.

1.4 Capillary electrophoresis

The scheme of a CE instrument is shown in Fig. 3. The instrumental consists of a hydrodynamic/electrokinetic sample introduction (not shown), a voltage supply, a detector, an electrode, a buffer reservoir, and a capillary. Upon applying voltage to the capillary the migration and separation occur depending on the electric charge of the analytes. Anions migrate to the anode while the cations migrate to cathode by their electrophoretic mobility (μ_{ep}). In untreated fused-silica capillaries, the electric double layer formed at the interface between the solid inner wall and the buffer solution (liquid phase) generates an electroosmotic flow (EOF) after an application of an electric field. The total mobility of analytes depends on the sum of the EOF and the (effective) electrophoretic mobilities of the analytes. The difference in mobility depends on charge density of compounds under specific conditions [39]. Under normal polarity of the applied voltage the EOF is directed toward the cathode.

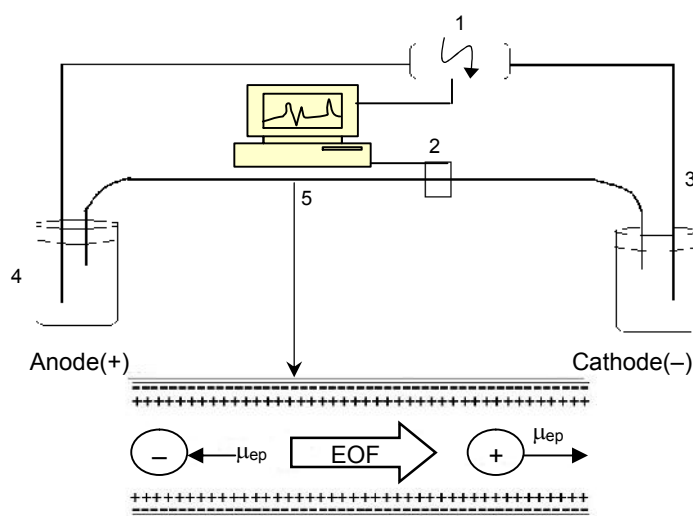


Figure 3. Schematic drawing of instrument CE consisting of a voltage supply (1), a detector (2), an electrode (3), a buffer reservoir (4), and a capillary (5). (modified from Ref. [39])

There are several modes and miscellaneous techniques of CE such as capillary zone electrophoresis (CZE), micellar electrokinetic chromatography (MEKC), microemulsion electrokinetic chromatography (MEEKC), capillary isoelectric focusing (CIEF), capillary gel electrophoresis (CGE), capillary isotachopheresis (CITP) and capillary electrochromatography (CEC). The modes used in this thesis are briefly discussed below;

1.4.1 Capillary zone electrophoresis (CZE)

CZE is essentially high voltage electrophoresis in free solution. The capillary is filled with the running buffer solution and the ionic analytes are separated under high electric fields (hundreds of volts per centimeter) on the basis of their electrophoretic mobility [25]. The electrophoretic migration velocity (v) of a charged analyte depends on its electrophoretic mobility (μ_e) and on the applied electric field (E):

$$v = \mu_e E \quad (1)$$

the μ_e is described by the following equation:

$$\mu_e = \frac{q}{6\pi\eta r} \quad (2)$$

with q = ion charge; r = ion radius; η = solution viscosity.

Besides the electrophoretic migration, electroosmosis (EOF) which is directed from the anodic to the cathodic end of an untreated fused-silica capillary is a fundamental factor in CZE. The electroosmosis mobility (μ_{EOF}) is described by the equation:

$$\mu_{EOF} = \frac{\varepsilon E \zeta}{4\pi\eta} \quad (3)$$

where ε = dielectric constant of the solvent, and ζ = zeta potential at the capillary wall.

The EOF is affected by experimental conditions, for example, buffer pH (affecting the dissociation of the wall silanols), ionic strength (affecting the zeta potential), organic solvents (affecting both zeta potential and buffer viscosity), and buffer additives (e.g. surfactants, methyl cellulose, polyacryamide, quaternary amines) [25].

1.4.2 Cyclodextrin-mediated capillary electrophoresis

Cyclodextrin-mediated CE is most widely used for the separation of enantiomers. There are several chiral selectors used for enantioseparation, such as cyclodextrins, chiral crown ethers, linear oligo- and polysaccharides, proteins, macrocyclic antibiotics, chiral calixarenes, chiral synthetic polymers, and molecularly imprinted polymers. However, cyclodextrins (CDs) with a wide variety of its derivatives are the most frequently used in chiral CE [40, 41]. Cyclodextrins are cyclic, non-reducing oligosaccharides consisting of D-glucopyranose units bonded through α -1,4-linkages. The smallest non-derivatized CD is the α -CD (containing six glucose units), followed by β -CD (seven glucose units), and γ -CD (eight glucose units), respectively. CDs have a hydrophobic interior cavity and a hydrophilic outside due to the hydroxyl groups. The structures of native α -, β - and γ -CD with a similar shape to a truncated cone are shown in Fig. 4. The narrow rim of the CDs has the primary hydroxyl groups at C6 and the wider rim contains the secondary hydroxyl groups at C2 and C3.

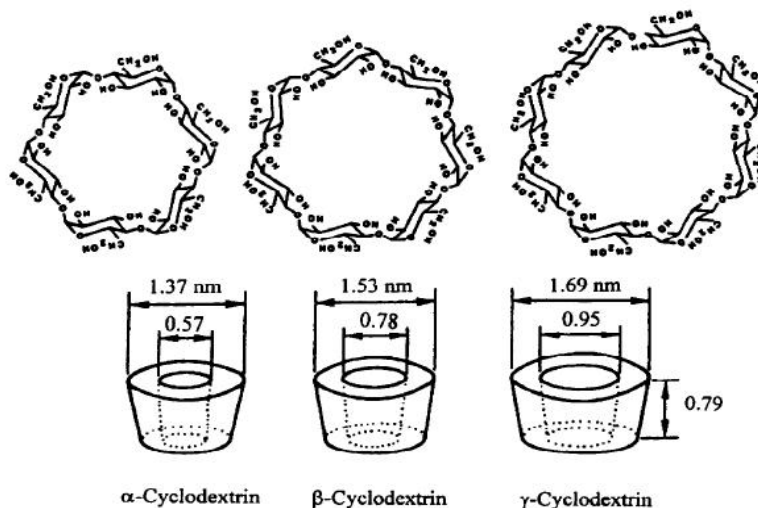
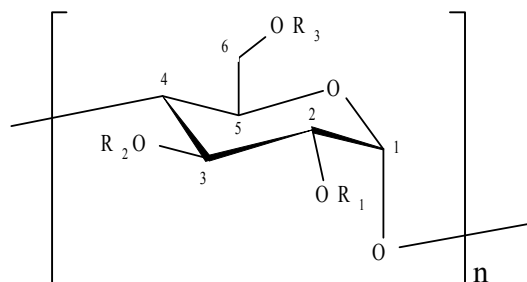


Figure 4. Molecular dimensions (d/nm) and structures of α -, β - and γ -CD (from Ref. [42])

Due to the limited capability of native CDs to resolve all enantiomers of interest CD derivatives have been increasingly used. Various functional groups have been utilized yielding neutral, negatively charged, positively charged, amphoteric and polymerized CD derivatives. Examples of commercial CD derivatives are listed in Table 6.

Table 6. Examples of commercially available cyclodextrins (modified from Ref. [43])



Derivatives	Substituents
<i>Native Cyclodextrins</i>	
α -cyclodextrin	H
β -cyclodextrin	H
γ -cyclodextrin	H
<i>Neutral CD derivatives</i>	
Methyl- α -cyclodextrin	CH ₃ , randomly substituted
Methyl- β -cyclodextrin	CH ₃ , randomly substituted
Heptakis-2,6-dimethyl- β -cyclodextrin	CH ₃ in positions 2 and 6
Heptakis-2,3,6-trimethyl- β -cyclodextrin	CH ₃ in positions 2, 3 and 6
Hydroxypropyl- α -cyclodextrin	CH ₂ -CH ₂ -CH ₂ -OH, randomly substituted
Hydroxypropyl- β -cyclodextrin	CH ₂ -CH ₂ -CH ₂ -OH, randomly substituted
Hydroxypropyl- γ -cyclodextrin	CH ₂ -CH ₂ -CH ₂ -OH, randomly substituted
<i>Negatively charged CD derivatives</i>	
Carboxymethyl- β -cyclodextrin	CH ₂ -COONa, randomly substituted
Sulfated α -cyclodextrin	SO ₃ Na, randomly substituted
Sulfated β -cyclodextrin	SO ₃ Na, randomly substituted
Sulfated γ -cyclodextrin	SO ₃ Na, randomly substituted
Sulfobutyl- β -cyclodextrin	CH ₂ -CH ₂ -CH ₂ -CH ₂ -SO ₃ Na, randomly substituted
Heptakis-6-sulfo- β -cyclodextrin	SO ₃ Na in position 6
Heptakis-(2,3-diacetyl-6-sulfo)- β -cyclodextrin	CH ₃ CO in positions 2 and 3, SO ₃ Na in position 6
Heptakis-(2,3-methyl-6-sulfo)- β -cyclodextrin	CH ₃ in positions 2 and 3, SO ₃ Na in position 6
<i>Positively charged CD derivatives</i>	
2-hydroxy-3-trimethylammoniopropyl- β -cyclodextrin	CH ₂ -CH(OH)-CH ₂ -N(CH ₃) ₃ Cl, randomly substituted
6-Monodeoxy-6-monoamino- β -cyclodextrin	NH ₂ instead of on 6-OH group

1.4.3 Theoretical background of chiral separations in capillary electrophoresis

Chiral recognition occurs due to the difference in the stereoconfigurations of enantiomers resulting in a different binding to the CDs. CDs containing a hydrophobic cavity are able to form inclusion complexes with analytes. Moreover, the hydroxyl groups or other substituents on the rim of CDs can also interact with the analytes. The temporary complexes between chiral selectors and enantiomers are stabilized by number of intermolecular interactions. The most important interactions are hydrogen bonding, $\pi - \pi$, dipole-dipole or ionic interactions. However, weaker interactions such as van der Waals interactions and charge transfer may also be important for chiral recognition mechanisms.

A theoretical model has been developed [44, 45] in order to explain the enantiomer separations in CE. In most cases it is assumed that there is a 1:1 interaction between a pair of enantiomers (R and S) and a chiral selector (C) as shown in Fig. 5.

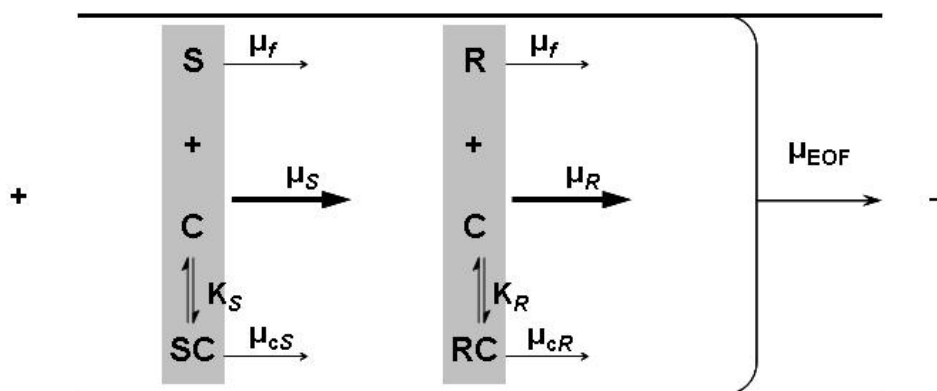


Figure 5. Schematic model of the complexation equilibrium between uncomplexed enantiomers, the chiral selectors and the enantiomer-chiral selector complexes.

The equation that allows the determination of the mobility difference, $\Delta\mu$, between two enantiomers can be written as follows [29, 44–46]:

$$\Delta\mu = \mu_R - \mu_S \quad (4)$$

or

$$\Delta\mu = \frac{\mu_f + \mu_{cR}K_R[C]}{1 + K_R[C]} - \frac{\mu_f + \mu_{cS}K_S[C]}{1 + K_S[C]} \quad (5)$$

where μ_R and μ_S are the observed mobilities of the R and S enantiomers, respectively, μ_f is the mobility of the analyte enantiomers in the uncomplexed form, K_R and K_S are the complexation equilibrium constants of the R and S enantiomers with the chiral selector, μ_{cR} and μ_{cS} are the mobilities of the respective temporary diastereomeric complexes between the chiral selector and the R and S enantiomers, and $[C]$ is the concentration of the chiral selector. While the mobilities of the uncomplexed enantiomers are identical, the equilibrium constants or the mobilities of temporary diastereomeric complexes may be different. The prerequisite for enantioseparations in CE is that

$$\Delta\mu \neq 0 \quad (6)$$

Regarding the equation 5, an enantioseparation in CE can be achieved either by the differences between the binding constants (or the affinities) of the enantiomers with a chiral selector ($K_R \neq K_S$) or the differences between the mobilities of the temporary diastereomeric complexes ($\mu_{cR} \neq \mu_{cS}$). A combination of both cases may also apply.

1.4.4 Microemulsion electrokinetic chromatography (MEEKC)

The microemulsions (MEs) are macroscopically homogeneous, optically transparent fluids which consist of more than one liquid phase. Generally, the microemulsion system is composed of an oil core or a lipophilic organic solvent, surfactants (such as sodium dodecyl sulfate (SDS)) which are used to stabilize the microemulsion droplet by lowering the surface tension between the two liquid phases, and/or co-surfactants (i.e. short-chain alcohol) which position themselves between the head groups of the surfactant molecules reducing the electrostatic repulsion. There are two principal types of MEs; an oil-in-water (O/W) ME representing the bulk phase that is made up by water and the oil are dispersed in water phase, while a water-in-oil (W/O) ME is where the bulk phase is made up by oil. In MEEKC microemulsion droplets are used as pseudostationary phases (PSPs). MEEKC was introduced for the separation of very lipophilic substances as well as hydrophilic substances as reviewed in [33, 47–49].

Chiral separations in MEEKC can be achieved by the use of chiral surfactants, chiral oil cores or by the addition of chiral selectors such as CDs to a microemulsion. The use of CDs in MEEKC also called “**Cyclodextrin-modified microemulsion electrokinetic chromatography (CD-modified MEEKC)**” can enhance the solubility of hydrophobic compounds, the enantioseparation of both charged and uncharged compounds as well as the resolution of the analytes. Fig. 6 shows the dual partitioning mechanisms between the CDs as a chiral selector and microemulsion droplets as a second pseudostationary phase resulting in a separation of both hydrophilic and hydrophobic compounds.

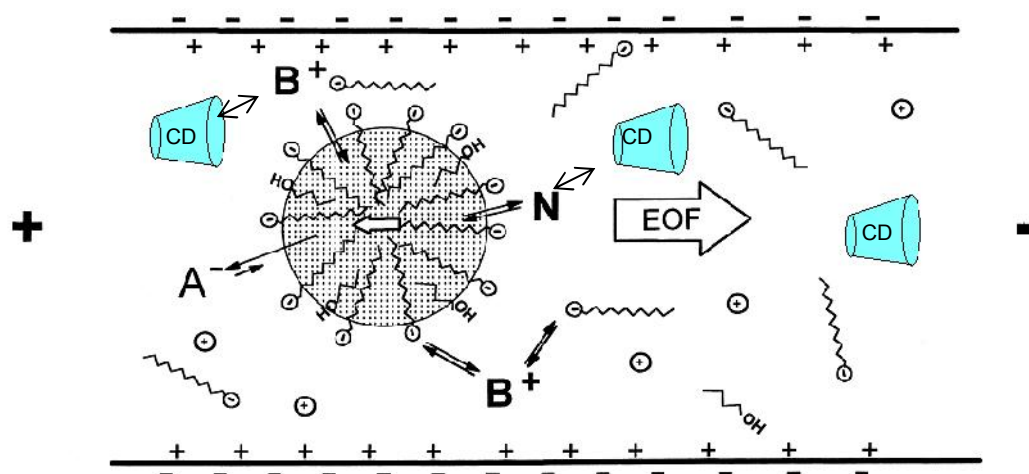


Figure 6. Schematic presentation of CD-modified MEEKC (modified from Ref. [50])

1.4.5 Factors affecting cyclodextrin-mediated stereoisomer separations

There are several factors that affect the stereoisomer separations such as CD type and concentration, pH and ionic strength of the background electrolyte (BGE), addition of organic solvents as well as temperature [51]. These factors are very important in order to achieve the enantioseparation and improve the enantioresolution. The factors are briefly described as follows.

➤ **Type of CD**

Most of CD derivatives are randomly substituted differing in the degree of substitution or in the position of the substituents. Therefore, the chiral recognition mechanism can hardly be predicted. Moreover, the problems related to batch-to-batch variability of CDs may occur with regard to poor reproducibility of the analytical methods.

➤ **CD concentration**

The influence of CD concentration on the enantioseparation of the analytes depends on the charge of both the CDs and the analytes. For example, the mobilities of the neutral analytes increase with the increasing of the charged CD concentration. While increasing the neutral CD concentration the mobilities of charged analytes are decelerated. In case of both analytes and CDs are charged the mobilities increase if both species carry the same charge, while a decrease of mobilities or a reversal of the migration order is observed if both species exhibit different charges.

➤ **The pH of background electrolyte (BGE) and ionic strength**

The pH of BGE containing ionizable CD derivatives determines the effective net charge and the effective mobility of the complexes. Moreover, the pH affects the ionization of analytes which can influence the interaction between CDs and analytes as well as between the capillary wall and analytes. Therefore, the pH will affect the migration times, the shape, and the efficiency of the analytes' peaks. The pH also affects the magnitude and the direction of the EOF which can affect the apparent selectivity and the enantioseparation.

An increase in ionic strength reduces the electromigration dispersion, resulting in higher separation efficiency. However, high BGE concentrations can lead to a high background current and possibly to excessive Joule heating which may lead to the loss of selectivity and efficiency.

➤ **The addition of organic solvents**

The presence of an organic solvent in the BGE can strongly influence the binding constant, the migration times, the BGE conductivity, and the solubility of both analytes and CDs [40]. Moreover, the addition of an organic solvent may affect the effective selectivity depending on the used CD concentration, for example, when the CD concentration is higher than its optimal value the addition of an organic solvent to decrease the complexation constants can consequently improve the selectivity values.

➤ **Temperature**

An increase in the temperature generally impairs the enantioselectivity [40]. However, unusual temperature effects on enantioseparations were also observed. In some cases an increase in temperature results in an improvement of enantioselectivity.

1.5 Scopes and aims

Enantiomers often differ in potency, pharmacological activity or toxicological properties due to their stereospecific interactions within biological systems. Consequently, single enantiomer drugs have been increasingly investigated and have become an important part of the overall pharmaceutical market. Thus, the safety and efficacy of a drug depend not only on the active ingredient itself but also on the impurities including enantiomeric impurities. Therefore, the control of enantiomeric purity as well as the impurity profiling of drug substances and products is an important concern of the pharmaceutical companies and regulatory authorities.

Traditionally, the enantiomeric purity is determined by optical rotation in pharmacopeias such as the European Pharmacopoeia or the United States Pharmacopoeia. However, this method is error prone especially in the case of a low specific rotation of the analyte. In modern monographs, chiral HPLC assays have been introduced for the determination of the enantiomeric composition while related substances are determined in an achiral HPLC test. In recent years CE has been recognized as a microanalytical technique that is able to separate closely related compounds as well as stereoisomers. Moreover, several CE modes are available allowing the analysis of essentially any analyte. Thus, the aim of the thesis was:

- Development and validation of CE assays for the simultaneous analysis of the enantiomeric purity of drug substances as well as the analysis of related substances. The single enantiomer drugs dexamphetamine and levodopa as well as the racemic drug chloroquine served as model compounds.
- Application of different modes of CE such as CZE and MEEKC for the chiral analysis.
- Application of the developed methods to commercial samples of the drugs.

CHAPTER II

MANUSCRIPTS

In Chapter II the following five publications are described. The impurity profiling of levoamphetamine and potential charged impurities in dexamphetamine sulfate was described in Manuscript I. In Manuscript II, a CE assay employing a dual CD system was developed for the simultaneous determination of charged and neutral impurities in dexamphetamine sulfate. An alternative CE assay employing CD-modified MEEKC for the impurity profiling of dexamphetamine sulfate was described in Manuscript III. The development and validation of a CE assay for determination of levodopa and the *R*-enantiomer as well as related substances were described in Manuscript IV. Manuscript V described the development and validation of a CE assay for the determination of enantiomeric purity of chloroquine enantiomers.

Overviews of manuscripts

Manuscript I

Profiling of levoamphetamine and related substances in dexamphetamine sulfate by capillary electrophoresis

Nino G. Kokiashvili, Sudaporn Wongwan, Carina Landgraf, Kristin Michaelis, Manuela Hammitzsch-Wiedemann, Gerhard K.E. Scriba, *Journal of Pharmaceutical and Biomedical Analysis* 2009, 50, 1050–1053.

In this publication a CE assay for the simultaneous determination of the enantiomeric purity of dexamphetamine as well as potential charged impurities, 1*R*,2*S*-(–)-norephedrine and 1*S*,2*S*-(+)-norpseudoephedrine, was developed and validated. Several native cyclodextrins as well as neutral and charged cyclodextrins such as β -CD, 2-hydroxypropyl- β -CD, 2,6-dimethyl- β -CD, 2,3,6-trimethyl- β -CD, carboxymethyl- β -CD, succinyl- β -CD, sulfated- β -CD and heptakis-(2,3-di-O-acetyl-6-O-sulfo)- β -CD (HDAS- β -CD) were evaluated. HDAS- β -CD was selected for the method development because of the better peak shape was obtained and the fact that HDAS- β -CD was a single isomer derivative, so that the batch-to-batch reproducibility will not affect the performance of the assay. The CD concentration, the buffer concentration and the buffer pH were subsequently varied in order to achieve acceptable resolutions and peak shapes of all analytes when the sample contained 2.0 mg/mL of dexamphetamine sulfate. Thus, the optimized background electrolyte consisted of a 100 mM phosphate buffer, pH 2.5, containing 10 mg/mL of HDAS- β -CD. The separation of the analytes was achieved in a 51/46.5 cm, 50 μ m id fused-silica capillary at a temperature 20°C and an applied voltage 25 kV. Detection was carried out at 205 nm. 1*R*,2*S*-(–)-ephedrine was used as internal standard. The optimized method was subsequently validated according to the ICH guideline Q2(R1) [52] with regard to linearity, range, limit of detection (LOD), limit of quantitation (LOQ) as well as intraday and interday precision. The LOQ of all analytes was 1.2 μ g/mL (0.06%) and the LOD was 0.4 μ g/mL (0.02%) for 1*R*,2*S*-(–)-norephedrine and 1*S*,2*S*-(+)-norpseudoephedrine, and 0.6 μ g/mL (0.03%) for levoamphetamine. Correlation coefficients of at least 0.995 were obtained and RSD values of intraday and interday precision were below 7% for all compounds. The assay was subsequently applied for the analysis of commercial dexamphetamine sulfate drug substances. Neither 1*R*,2*S*-(–)-norephedrine nor 1*S*,2*S*-(+)-norpseudoephedrine could be detected while levoamphetamine at concentrations between 2.8% and 4.0% was found in all samples. The studies indicated that the drug substances were obtained by fractionated crystallization of racemic amphetamine. The present stereoselective CE assay was also compared to the determination of optical rotation according to the pharmacopeial tests of the USP 32 [53] and the BP 2009 [54] by investigating enantiomeric purity of the same dexamphetamine sulfate sample.

Manuscript II

CE assay for simultaneous determination of charged and neutral impurities in dexamphetamine sulfate using a dual CD system

Sudaporn Wongwan, Bunleu Sungthong, Gerhard K.E. Scriba, *Electrophoresis* 2010, 31, 1475–1481.

The objective of the study was to develop and validate a stereoselective CE assay for the simultaneous determination of both charged and neutral impurities in dexamphetamine sulfate. As none of the charged impurities could be detected in commercial samples (see manuscript I), it was hypothesized that dexamphetamine was synthesized from phenylacetone (1-phenyl-2-propanone, benzyl methyl ketone) via Leuckart reaction or by reduction of the oxime. Various negatively charged CDs, e.g., sulfated α -CD, sulfated β -CD, sulfated γ -CD, heptakis-(2,3-di-O-acetyl-6-O-sulfo)- β -CD, carboxymethyl- β -CD, carboxymethyl- γ -CD, sulfopropyl- β -CD, sulfopropyl- γ -CD and sulfobutylether- β -CD (DS 5) were initially investigated for their ability to separate the enantiomers of amphetamine and the neutral impurities. High enantioresolution of amphetamine could be achieved by employing sulfated β -CD, but the neutral analytes migrated slowly and the peaks were rather broad. A baseline separation with an acceptable peak shape was achieved by applying SBE(V)- β -CD in the BGE. However, the resolution of enantiomers of amphetamine was poorer than the sulfated β -CD. Therefore, a dual CD system comprising sulfated β -CD for the effective resolution of the amphetamine enantiomers and SBE(V)- β -CD for the separation of phenylacetone and phenylacetone oxime was selected. The effect of CD concentrations as well as the degree of substitution (DS) of sulfobutylether- β -CD (DS = 4, 5 and 7) to the peak separation and peak shape was studied. Besides, the effective length of the capillary, the applied voltage and the buffer pH were subsequently optimized. The optimized conditions employed a 50 mM phosphate buffer, pH 3.0, containing 80 mg/mL of SBE(V)- β -CD and 25 mg/mL sulfated β -CD as background electrolyte, a 50 μ m id fused-silica capillary with an effective length of 35 cm (total length 40.2 cm) at an applied voltage –10 kV operated at 20°C. The optimized assay also allowed the separation of the *E,Z*-stereoisomers of phenylacetone oxime. The optimized method was subsequently validated according to the ICH guideline Q2(R1) [52]. 1*R*,2*S*-(–)-ephedrine was used as internal standard. The LOQ was 0.05% (2.5 μ g/mL) for all potential impurities. Correlation coefficients of at least 0.993 were observed and RSD values of intraday and interday precision were below 8% for all compounds. The method was subsequently applied to the analysis of commercial samples of dexamphetamine sulfate. Levoamphetamine could be detected in all samples at concentrations between 3.2% and 3.7%. Neither 1*R*,2*S*-(–)-norephedrine nor 1*S*,2*S*-(+)-norpseudoephedrine could be detected. In contrast, peaks corresponding to phenylacetone and both isomers of *E,Z*-phenylacetone oxime were observed indicating the synthetic origin of the investigated drug substances.

Manuscript III

Impurity profiling of dexamphetamine sulfate by cyclodextrin-modified microemulsion electrokinetic chromatography

Sudaporn Wongwan, Gerhard K.E. Scriba, *Electrophoresis* 2010, 31, 3006–3011.

Since the simultaneous application of MEEKC to the determination of achiral impurities of drugs and the analysis of enantiomeric impurities has not been reported to date, the study was conducted in order to evaluate CD-modified MEEKC for the determination of stereoisomeric purity as well as impurity profiling of drugs. Dexamphetamine sulfate and its impurities were selected as an example due to the diversity of analytes comprising charged and uncharged analytes, chiral and achiral analytes and a pair of enantiomers as well as another pair of *E*- and *Z*-geometrical isomers. The microemulsion containing sodium dodecyl sulfate (SDS), ethyl acetate, 1-butanol, 2-propanol and a 50 mM sodium phosphate buffer was evaluated. Sulfated β -CD was initially selected as the chiral selector due to its wide commercial availability and a relatively inexpensive. Composition of the microemulsion, the CD concentration, the buffer concentration, the buffer pH and the applied voltage were varied in order to achieve the baseline separation of all analytes. Moreover, the effect of SDS, ethyl acetate, 1-butanol, 2-propanol and sulfated β -CD on peak resolution and peak shape was investigated. During method development, it was observed that preparation of the microemulsion affected the separation between dexamphetamine and levoamphetamine as well as between phenylacetone *E*-oxime and 1*S*,2*S*-(+)-norseudoephedrine depending on the addition of sulfated β -CD either before or after the preparation of the microemulsion. Amino acids and their derivatives as well as structurally not related compounds, carbamazepine, were screened. Finally carbamazepine was selected as an internal standard due to the peak shape and short migration time. The optimized method employed a background electrolyte containing 1.5% w/w SDS, 0.5% w/w ethyl acetate, 3.5% w/w 1-butanol, 2.5% w/w 2-propanol and 92% w/w 50 mM phosphate buffer, pH 3.0, containing 5.5% w/w sulfated β -CD. The separations were carried out in a 50 μ m id fused-silica capillary with an effective length of 40 cm (total length 50.2 cm) at an applied voltage –14 kV and at a temperature of 20°C. Subsequently, the method was validated according to the ICH guideline Q2(R1) [52]. The LOQ estimated at a signal-to-noise ratio of 10 was 0.1% (3 μ g/mL) for all potential impurities except for the *Z*-oxime with a LOQ of 0.5% (15 μ g/mL). Correlation coefficients of at least 0.994 were observed and RSD values of intraday and interday precision were below 8.2% for all compounds. Four commercial samples of dexamphetamine were subsequently analyzed employing the validated method. Levoamphetamine was detected in all samples at concentrations between 3.2% and 3.8%, whereas none of the other impurities could be detected.

Manuscript IV

Determination of related substances of levodopa including the *R*-enantiomer by CE

Sudaporn Wongwan, Manuela Hammitzsch-Wiedemann, Gerhard K.E. Scriba, *Electrophoresis* 2009, 30, 3891–3896.

Levodopa is described in monographs in the European Pharmacopoeia [55] and the United State Pharmacopoeia (USP) [53]. The related substances specified by both pharmacopeias include *L*-tyrosine, 3-methoxy-*L*-tyrosine, and 6-hydroxy-DOPA (6-OH-DOPA). In the European Pharmacopoeia, different HPLC assays are required for the determination of related substances and enantiomeric purity, while the USP prescribed a HPLC assay for the determination of related substances and a polarimetric method for the determination of enantiomeric purity. However, as none of the simultaneous assay had been reported, a stereoselective CE assay for the simultaneous determination of related substances as well as the enantiomers of DOPA was developed and validated. Sulfated β -CD was selected as chiral selector in the present study. The background electrolyte containing 2 mg/mL, 4 mg/mL, 6 mg/mL and 8 mg/mL sulfated β -CD and a 0.12 M sodium phosphate buffer, pH 2.0, was evaluated. Subsequently, the buffer concentration, buffer pH, applied voltage and effective length of the capillary were varied in order to achieve the baseline separation of all analytes as well as acceptable peak shapes and short migration times. It was observed that substituting the sodium phosphate buffer with a phosphate buffer prepared from orthophosphoric acid and sodium hydroxide solution the buffer molarity and the concentration of sulfated β -CD could be reduced with a comparable peak resolution. Due to the fact that sulfated β -CD used in this study is a randomly substituted CD derivative which may hamper the reproducibility of the assay, other two different batches from the other suppliers were investigated. Clearly, different batches of sulfated β -CD affected the separation, especially the critical resolution between levodopa and *L*-tyrosine. The optimized conditions employed a 0.1 M phosphate buffer, pH 2.0, prepared from orthophosphoric acid and sodium hydroxide solution containing 6 mg/mL sulfated β -CD as background electrolyte, a 50 μ m id fused-silica capillary with an effective length of 35 cm (total length 45.2 cm), an applied voltage of 20 kV and 18°C capillary temperature. The optimized conditions also allowed the separation of the enantiomers of 6-OH-DOPA. The optimized method was subsequently validated according to the ICH guideline Q2(R1) [52]. *L*-Phenylalanine was used as internal standard. The LOQ was 0.1% (2 μ g/mL) for all impurities. Correlation coefficients of at least 0.990 were observed and RSD values of intraday and interday precision were below 10% for all compounds. Three samples of commercial levodopa drug substance were subsequently analyzed. Neither 6-OH-DOPA nor 3-methoxy-*L*-tyrosine could be detected in all samples, whereas *L*-tyrosine was detected in all samples but the quantities were below the LOQ. *D*-DOPA could be detected in two samples, sample one had a quantity of *D*-DOPA below the LOQ, while another one which is the reference substance of the European Pharmacopoeia an amount of *D*-DOPA up to $0.15 \pm 0.01\%$ could be detected.

Manuscript V

Development and validation of a capillary electrophoresis assay for the determination of stereoisomeric purity of chloroquine enantiomers

Sudaporn Wongwan, Gerhard K.E. Scriba, *submitted manuscript*.

Chloroquine is commercially available in a racemic mixture. The study of stereoselective biological activities as well as toxicities of chloroquine enantiomers is still incomplete. Therefore, the availability of pure enantiomers of (+)- and (–)-chloroquine is important. A stereoselective CE assay was developed and validated in order to determine the enantiomeric purity of *R*-(–)-chloroquine substance. Initial background electrolytes containing 5 mg/mL, 10 mg/mL, 20 mg/mL, and 30 mg/mL sulfated β -CD and a 50 mM phosphate buffer, pH 2.5, were evaluated. Applying normal polarity none of analytes could be detected. In contrast, all analytes could be detected with reversed polarity of the applied voltage. However, the peaks were rather broad. Subsequently, the buffer concentration was increased from 50 mM to 100 mM resulting in sharp peaks. Upon injecting a sample containing 2 mg/mL of *R*-(–)-chloroquine and 20 μ g/mL of *S*-(+)-chloroquine peak deformation of *S*-(+)-chloroquine was detected. Thus, background electrolytes containing SBE(V)- β -CD (DS \approx 4.9) and SBE(VII)- β -CD (DS \approx 6.6) were subsequently investigated. The separation as well as acceptable peak shapes could be achieved by employing the background electrolyte containing 30 mg/mL SBE(VII)- β -CD and the reversed polarity, whereas SBE(V)- β -CD was insufficient. Migration order reversal of chloroquine enantiomers was observed when sulfated β -CD was substituted with SBE(VII)- β -CD. The optimized method employed a 100 mM sodium phosphate buffer, pH 2.5, containing 30 mg/mL SBE(VII)- β -CD as the background electrolyte, a 50 μ m id uncoated fused-silica capillary with an effective length of 40 cm (total length 50.2 cm), an applied voltage of -25 kV and 20°C capillary temperature. The optimized method was subsequently validated according to the ICH guideline Q2(R1) [52]. Carbamazepine was used as internal standard. Method validation was performed with racemic chloroquine because enantiomerically pure chloroquine was not available. The LOQ was 0.05% (1.5 μ g/mL) for individual enantiomers based on a concentration of 3 mg/mL of a major compound. Correlation coefficients of at least 0.997 were observed and RSD values of intraday and interday precision were below 6% for all compounds. The sample of *R*-(–)-chloroquine substance was analyzed using the validated method. *S*-(+)-chloroquine at levels 0.24% \pm 0.01% (means \pm SD) was observed in *R*-(–)-chloroquine. Moreover, an unknown peak which migrated faster than the enantiomers was also detected at 0.4% level computed from the normalized peak area.

Manuscript I

Profiling of levoamphetamine and related substances in dexamphetamine sulfate by capillary electrophoresis

Nino G. Kokiashvili, Sudaporn Wongwan, Carina Landgraf, Kristin Michaelis, Manuela Hammitzsch-Wiedemann, Gerhard K.E. Scriba, *Journal of Pharmaceutical and Biomedical Analysis* 2009, 50, 1050–1053.



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Short communication

Profiling of levoamphetamine and related substances in dexamphetamine sulfate by capillary electrophoresis

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ABSTRACT

A capillary electrophoresis method for the simultaneous determination of the enantiomeric purity of dexamphetamine as well as the analysis of 1R,2S-(–)-norephedrine and 1S,2S-(+)-norpseudoephedrine as potential impurities has been developed and validated. Heptakis-(2,3-di-O-acetyl-6-O-sulfo)- β -cyclodextrin was chosen as chiral selector upon a screening of neutral and charged cyclodextrin derivatives. Separation of the analytes was achieved in a fused-silica capillary at 20 °C using an applied voltage of 25 kV. The optimized background electrolyte consisted of a 0.1 M sodium phosphate buffer, pH 2.5, containing 10 mg/ml of the cyclodextrin. The assay was linear in the range of 0.06–5.0% of the impurities based on a concentration of 2.0 mg/ml dexamphetamine sulfate in the sample solution. Analysis of commercial dexamphetamine sulfate samples revealed the presence of 3–4% of levoamphetamine while norephedrine or norpseudoephedrine could not be detected, indicating that the compound was prepared by fractionated crystallization of racemic amphetamine. Comparison with polarimetric measurements indicated that dexamphetamine with an enantiomeric excess as low as 80% still passes the pharmacopeial test of specific rotation while an amount of 0.06% of levoamphetamine can be detected by capillary electrophoresis.

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1. Introduction

Dexamphetamine (dextroamphetamine, 2S-(+)-amphetamine, (S)-1-phenyl-2-propanamine, Fig. 1) is the dextrorotary (S)-enantiomer of the racemic drug amphetamine. Both, dexamphetamine and amphetamine are controlled substances in most European countries and the United States of America. Dexamphetamine is a central stimulant therapeutically used in the treatment of narcolepsy [1] and the attention deficit hyperactivity disorder (ADHD) of children [2]. The effect in ADHD is believed to be mediated via several mechanisms including the binding of the drug to the pre-synaptic dopamine transporter inducing a reversed transport process as well as stimulation of pre-synaptic inhibitory autoreceptors resulting in reduced activity in dopaminergic and noradrenergic pathways [3].

Dexamphetamine sulfate is described in monographs by the United States Pharmacopoeia 32 (USP 32) [4] and the British Pharmacopoeia 2009 (BP 2009) [5]. Both pharmacopoeias determine the stereoisomeric purity of the drug by optical rotation. The USP states

a specific rotation value $[\alpha]_D^{25} = 20\text{--}23.5^\circ$ ($c=4$ in water), the BP 2009 specifies $[\alpha]_D^{25} = 19.5\text{--}22^\circ$ ($c=8$ in water). In addition, the USP determines the chromatographic purity limiting individual impurities to 0.1% and the sum to 0.5%. No impurities are specified. The BP 2009 does not prescribe such a test. As the optical rotation is rather low and, therefore, prone to low sensitivity with regard to the detection of the R-enantiomer several other techniques have been applied to the determination of the enantiomeric purity of dexamphetamine drug substance. These include enhancement of the optical rotation upon derivatization [6], NMR using europium shift reagents [7], complexation by heptakis-(2,3-di-O-acetyl)- β -cyclodextrin [8,9] or derivatization with 1R-(–)-myrtenal [10] as well as chromatographic techniques [11–13].

Capillary electrophoresis (CE) has been recognized as a suitable technique for the determination of the stereoisomeric purity of drugs [14–22] as well as the analysis of related substances [16,17,19,23]. Moreover, it has been demonstrated that CE can be used to determine chiral impurities as well as (achiral) related substances simultaneously [24,25]. Many publications have described the enantioseparation of amphetamine and other phenylalkylamines by CE, for example [26–33]. Only one study has employed CE for the determination of the enantiomeric composition of amphetamine in comparison to NMR upon complexation

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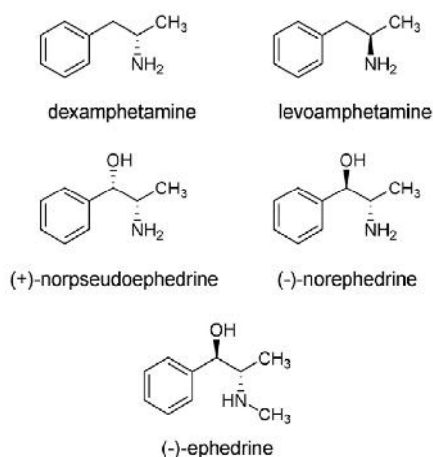


Fig. 1. Structures of dexamphetamine, levoamphetamine, 1R,2S-(+)-norpseudoephedrine and the internal standard 1R,2S-(+)-ephedrine.

with heptakis-(2,3-di-O-acetyl)- β -cyclodextrin [9]. No study has attempted the simultaneous determination of related substances and the enantiomeric purity of dexamphetamine. In the present study 1R,2S-(+)-norpseudoephedrine and 1S,2S-(-)-norpseudoephedrine were studied as related substances because dexamphetamine can be prepared from these natural compounds [34,35].

2. Experimental

2.1. Chemicals

All chemicals were of analytical grade. Dexamphetamine sulfate and 1R,2S-(+)-norpseudoephedrine hydrochloride were from Fagron GmbH (Barsbüttel, Germany), racemic amphetamine sulfate and 1R,2S-(+)-ephedrine hydrochloride were from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany), and 1S,2S-(-)-norpseudoephedrine hydrochloride was from Hünslers AG (Herisau, Switzerland). Heptakis-(2,3-di-O-acetyl-6-O-sulfo)- β -cyclodextrin (HDAS- β -CD) was obtained from Regis Technologies (Norton Grove, IL, USA). Sodium hydroxide solution was from Fisher Scientific (Schwerte, Germany) and phosphoric acid was from Carl Roth GmbH (Karlsruhe, Germany). All buffers and solutions were prepared in deionized, double-distilled water.

2.2. Instrumentation

All analyses were carried out on a BioFocus 3000 instrument (Biorad, Munich, Germany) equipped with a diode array detector using 50 μ m I.D., 375 μ m O.D. fused-silica capillaries (BGB Analytik, Schloßböckelheim, Germany). The total length of the capillary was 51 cm with an effective length of 46.5 cm. Detection was carried out at 205 nm. The optimized background electrolyte consisted of 0.1 M phosphate buffer, pH 2.5, prepared from 0.1 M phosphoric acid by adjusting the pH with 1 M sodium hydroxide solution. HDAS- β -CD at a concentration of 10 mg/ml was dissolved in the buffer after the adjustment of the pH. The capillary was operated at 20 °C. The applied voltage was 25 kV.

A new capillary was treated with 1 M sodium hydroxide for 10 min, 0.1 M sodium hydroxide for 20 min, 0.1 M phosphoric acid and water for 10 min each. At the beginning of each day, the capillary was rinsed with water, 0.1 M sodium hydroxide and 0.1 M phosphoric acid for 5 min each followed by flushing with water for

10 min. Between the injections, the capillary was washed subsequently with 0.1 M sodium hydroxide for 1 min and water for 2 min followed by a rinse with the background electrolyte for 5 min. At the end of the day, the capillary was flushed with water for 2 min, 0.1 M sodium hydroxide for 10 min, water for 1 min, 0.1 M phosphoric acid for 5 min and water for 10 min. Samples were introduced by hydrodynamic injection at 0.5 psi for 2 s.

Polarimetric measurements were carried out on a Polarotronic E instrument (Schmidt & Haensch, Berlin, Germany) at a wavelength of 589.3 nm using a 10 cm standard cuvette thermostated at 20 \pm 1 °C.

2.3. Method validation

The assay was validated for concentrations corresponding to a range of 0.06–5.0% for levoamphetamine (1.2–100 μ g/ml racemic amphetamine) and 0.06–5.0% of (-)-norephedrine and (+)-norpseudoephedrine (1.2–100 μ g/ml) based on a final concentration of 2.0 mg/ml dexamphetamine sulfate. Method validation was conducted according to ICH guideline Q2(R1) [36] with regard to range, linearity, limit of detection and quantitation, and precision. Linearity was estimated by unweighted linear regression using the least square method. Detection and quantitation limit were based on a signal-to-noise ratio of 3:1 and 10:1, respectively. Precision was determined at a low concentration (0.1%) and a high concentration (3.0%). Intraday precision was calculated from six replicate injections on the same day while interday precision was based on six injections on three consecutive days.

3. Results and discussion

3.1. Method development

The CE separation of the amphetamine enantiomers has been reported in several studies using native cyclodextrins (CDs) as well as neutral and charged CD derivatives as chiral selectors [9,26–33]. Thus, an initial screening was performed in a 50 mM phosphate buffer, pH 2.5, evaluating β -CD, 2-hydroxypropyl- β -CD, 2,6-dimethyl- β -CD, 2,3,6-trimethyl- β -CD, carboxymethyl- β -CD, succinyl- β -CD, sulfated β -CD and HDAS- β -CD as chiral selectors. Good resolution of $R_s > 2$ was obtained with sulfated β -CD and HDAS- β -CD at concentrations of 1–3 mg/ml. Subsequently, HDAS- β -CD was selected for method development due to better peak shape and the fact that the CD is a single isomer derivative so that batch to batch reproducibility will not affect the performance of the analytical method as it may be the case with randomly substituted CD derivatives. Increasing buffer pH to 3.5 and 6.5 led to shorter migration times but also poorer peak shape. Thus, pH 2.5 was considered optimal. Injecting dexamphetamine at a concentration of 2.0 mg/ml led to peak deformation of the later migrating levoamphetamine. Increasing the concentration of HDAS- β -CD to 10 mg/ml and the buffer concentration to 0.1 M resulted in acceptable peak shapes of all analytes with an analysis time of less than 15 min (Fig. 2A). Thus, the optimized background electrolyte consisted of a 0.1 M phosphate buffer, pH 2.5, containing 10 mg/ml of HDAS- β -CD. At an applied voltage of 25 kV a current of about 90 μ A was observed which was considered acceptable.

3.2. Method validation

The optimized method was validated according to the ICH guideline Q2(R1) [36] with regard to linearity, range, limit of detection (LOD), limit of quantitation (LOQ) as well as intraday and interday precision. Because enantiomerically pure dexamphetamine was not available, method validation was performed

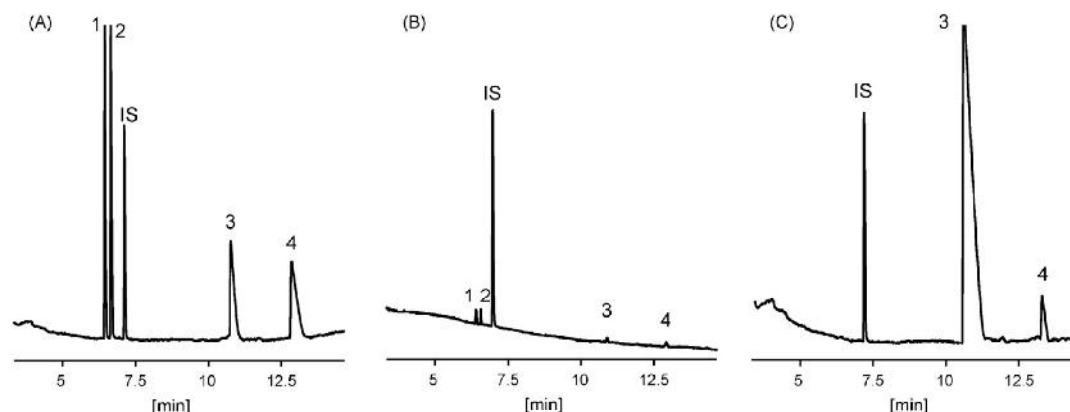


Fig. 2. Electropherograms of (A) separation of standards (75–144 µg/ml), (B) standards at the 0.1% (norpseudoephedrine and norephedrine) and 0.06% level (dexamphetamine and levoamphetamine) with respect to a concentration of 2 mg/ml dexamphetamine sulfate containing 75 µg/ml of ephedrine and (C) commercial sample of 2.0 mg/ml dexamphetamine sulfate containing 3.79% levoamphetamine, (1) 1S,2S-(+)-norpseudoephedrine, (2) 1R,2S-(+)-norephedrine, (3) dexamphetamine, (4) levoamphetamine. Experimental conditions: 0.1 M sodium phosphate buffer, pH 2.5, containing 10 mg/ml HDAS β CD; 51/46.5 cm, 50 µM i.d. fused silica capillary; 25 kV; 20 °C.

Table 1
Calibration data of levoamphetamine, 1S,2S-(+)-norpseudoephedrine and 1R,2S-(+)-norephedrine.

Compound	Range (%) ^a	Slope ± SD	Intercept ± SD	Correlation coefficient (r^2)	Intraday precision		Interday precision	
					0.1%	3.0%	0.1%	3.0%
1S,2S-(+)-Norpseudoephedrine	0.06–5.0	0.233 ± 0.002	0.004 ± 0.006	0.9984	2.78	2.51	3.21	1.81
1R,2S-(+)-Norephedrine	0.06–5.0	0.233 ± 0.005	0.001 ± 0.014	0.9960	3.76	1.05	4.54	2.01
Levoamphetamine	0.06–5.0	0.219 ± 0.005	−0.004 ± 0.014	0.9954	6.54	3.07	6.70	5.33

^a Corresponding to a concentration of 2 mg/ml dexamphetamine sulfate.

with racemic amphetamine. Levoamphetamine, norephedrine and norpseudoephedrine were analyzed over a range of 1.2–100 µg/ml corresponding to 0.06–5.0% of the impurities based on a concentration of 2.0 mg/ml dexamphetamine sulfate. Ephedrine at a concentration of 75 µg/ml was used as internal standard to compensate for injection errors and minor fluctuations of the migration time. Calibration data obtained by unweighted linear regression are summarized in Table 1. Correlation coefficients of at least 0.99 were observed indicating sufficient linearity in the investigated concentration range. The 95% confidence intervals of the y-intercepts included zero for all compounds so that a systematic error can be excluded. The LOQ estimated at a signal-to-noise ratio of 10 was 1.2 µg/ml (0.06%) for all compounds and the LOD corresponding to a signal-to-noise ratio of 3 was 0.4 µg/ml (0.02%) for norephedrine and norpseudoephedrine and 0.6 µg/ml (0.03%) for levoamphetamine. Fig. 2B shows an electropherogram of norephedrine and norpseudoephedrine at the 0.1% level as well as dexamphetamine and levoamphetamine at the 0.06% level. The RSD values of intraday and interday precision at 2 µg/ml (0.1%) and 60 µg/ml (3%) were below 7% for all compounds (Table 1).

3.3. Method application

The assay was subsequently applied for the analysis of commercial dexamphetamine sulfate drug substance. All samples were investigated at a concentration of 2.0 mg/ml containing 75 µg/ml of the internal standard. Neither norephedrine nor norpseudoephedrine could be detected in the samples. The content of levoamphetamine varied between 2.8 and 4.0% (Fig. 2C, Table 2). In addition to analysis via the peak area ratio, peak area normalization was also applied. Comparable concentrations of levoamphetamine were determined by both methods. The data indicate that the dex-

amphetamine sulfate batches investigated were not synthesized starting from the natural compounds norephedrine or norpseudoephedrine but were rather obtained by synthetic procedures leading to racemic amphetamine such as reductive amination starting from phenyl-2-propanone. The racemic amphetamine is subsequently resolved by fractionated crystallization using optically active acids such as (2S,3S)-(-)-tartaric acid [37]. Our data are in accordance with published data reporting amounts of levoamphetamine between 1.2 and 9.3% in dexamphetamine bulk drug and pharmaceutical preparations [11,12].

Only few of the above cited studies on the determination of the enantiomeric purity of dexamphetamine reported LOQ data. Using chiral europium shift reagents 5% of levoamphetamine could be determined by NMR [7]. Ihunhorst et al. were able to determine 1.25% enantiomeric impurity in dexamphetamine by NMR using heptakis(2,3-di-O-acetyl)- β -CD as shift reagent [8,9]. The CD was also applied in an enantioselective CE assay but inconclusive data were obtained compared to the application in NMR [9]. A LOQ of 0.5% was reported for a HPLC assay employing a chiral stationary phase. The present CE assay allowed the determination of levoamphetamine down to the 0.06% level. Moreover, related substances can be simultaneously analyzed.

Table 2
Content of levoamphetamine in commercial samples of dexamphetamine drug substance (mean ± SD, $n = 3$).

Sample	[%] peak area ratio	[%] peak area normalization
1	3.79 ± 0.12	4.13 ± 0.22
2	2.79 ± 0.23	2.89 ± 0.13
3	3.97 ± 0.15	4.03 ± 0.05
4	2.88 ± 0.16	3.04 ± 0.35
5	3.26 ± 0.24	2.98 ± 0.26

Dexamphetamine sulfate sample 1 was also investigated by polarimetry according to the pharmacopeial tests of the USP 32 [4] and the BP 2009 [5]. A specific rotation $[\alpha]_D^{20} = 21.5 \pm 0.43^\circ$ ($n = 3$) was found. The addition of 2% and 4% racemic amphetamine resulted in values of $21.3 \pm 0.13^\circ$ and $21.1 \pm 0.17^\circ$, respectively. Considering the levoamphetamine content of sample 1 of 3.79% determined by CE, the optical rotation values correspond to an enantiomeric excess (ee) of 92.4%, 90.6% and 88.9%, respectively. From these values it can be estimated that a dexamphetamine sample with an ee as low as approximately 80%, i.e. containing 10% of levoamphetamine, would yield an $[\alpha]_D^{20}$ value of 20° still passing the test of specific optical rotation of the USP 32 ($[\alpha]_D^{25} = 20\text{--}23.5^\circ$) or the BP 2009 ($[\alpha]_D^{25} = 19.5\text{--}22^\circ$). This clearly indicates the limitations of the determination of the specific rotation of a dexamphetamine sample for the analysis of the stereochemical purity of the drug.

4. Conclusions

A CE method has been developed and validated for the simultaneous determination of some potential impurities of dexamphetamine as well as the stereochemical purity of dexamphetamine. The method allowed the determination of the impurities at the 0.06% level. While the related compounds could not be detected in commercial samples of dexamphetamine sulfate, a content of 3–4% of levoamphetamine was generally found. This indicated that the drug was obtained by fractionated crystallization of racemic amphetamine. Compared with the measurement of the optical rotation the stereoselective CE assay proved to be superior as a dexamphetamine sample with an enantiomeric excess of as low as 80% still met the criteria of the test of the optical rotation of the USP 32 and the BP 2009. As CE is capable to simultaneously analyze related substances and the stereochemical purity of drugs such methods may be preferable to enantioselective chromatographic methods or other methods reported in the literature.

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Manuscript II

CE assay for simultaneous determination of charged and neutral impurities in dexamphetamine sulfate using a dual CD system

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Research Article

CE assay for simultaneous determination of charged and neutral impurities in dexamphetamine sulfate using a dual CD system*

A CE assay for the simultaneous determination of charged and uncharged potential impurities (1*S*,2*S*-(+)-norpseudoephedrine, 1*R*,2*S*-(–)-norephedrine, phenylacetone and phenylacetone oxime) of dexamphetamine sulfate including the stereoisomer levoamphetamine was developed and validated. The optimized background electrolyte consisted of a 50 mM sodium phosphate buffer, pH 3.0, containing 80 mg/mL sulfobutylether- β -CD and 25 mg/mL sulfated β -CD. Separations were performed in 40.2/35 cm, 50 μ m id fused-silica capillaries at a temperature of 20°C and an applied voltage of –10 kV. 1*R*,2*S*-(–)-ephedrine was used as internal standard. The assay was validated in the range of 0.05–1.0% for the related substances and in the range of 0.05–5.0% for levoamphetamine. The LOD was 0.01–0.02% depending on the analyte. The assay also allowed the separation of the *E*,*Z*-stereoisomers of phenylacetone oxime. The effect of the degree of substitution of sulfobutylether- β -CD was investigated. In commercial samples of dexamphetamine sulfate between 3.2 and 3.7% of levoamphetamine were found. Furthermore, phenylacetone and phenylacetone oxime could be observed at the LOD, indicating the synthetic origin of the investigated samples.

Keywords:

CE / CD / Dexamphetamine enantioseparation / Impurity profiling

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1 Introduction

Dexamphetamine (dextroamphetamine, 2*S*-(+)-amphetamine, (*S*)-1-phenyl-2-propanamine, Fig. 1) is the dextrorotatory (*S*)-enantiomer of the racemic drug amphetamine. Both dexamphetamine and amphetamine are controlled substances in most European countries and the United States of America. Dexamphetamine is a central stimulant therapeutically used in the treatment of the attention deficit hyperactivity disorder of children [1, 2] and narcolepsy [3]. The effect in attention deficit hyperactivity disorder is believed to be mediated via several mechanisms including the binding of the drug to the pre-synaptic dopamine transporter inducing a reversed transport process as well as stimulation of pre-synaptic inhibitory autoreceptors, result-

ing in reduced activity in dopaminergic and noradrenergic pathways [4].

Dexamphetamine can be synthesized by various methods, for example, starting from chiral precursors such as *D*-phenylalanine [5] or from chiral β -hydroxy-phenylethylamines such as 1*R*,2*S*-(–)-norephedrine or 1*S*,2*S*-(+)-norpseudoephedrine [6, 7]. Alternatively, the compound can be obtained from racemic amphetamine synthesized from phenylacetone (1-phenyl-2-propanone, benzyl methyl ketone) by Leuckart reaction or by reduction of the oxime [8, 9] followed by fractional crystallization with *L*-(+)-tartaric acid [10]. Thus, charged as well as neutral starting materials or synthetic by-products as well as the enantiomer levoamphetamine (2*R*-(–)-amphetamine) may be present as related substances in the drug.

Dexamphetamine sulfate is described in monographs by the United States Pharmacopeia 32 (USP 32) [11] and the British Pharmacopocia 2009 (BP 2009) [12]. The USP determines the chromatographic purity limiting individual impurities to 0.1% and the sum to 0.5%. No impurities are specified. The BP 2009 does not prescribe such a test. Moreover, both pharmacopeias determine the

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Abbreviations: DS, degree of substitution; SBE- β -CD, sulfobutylether- β -CD

*This work is dedicated to Professor Salvatore Fanali on the occasion of his 60th birthday.

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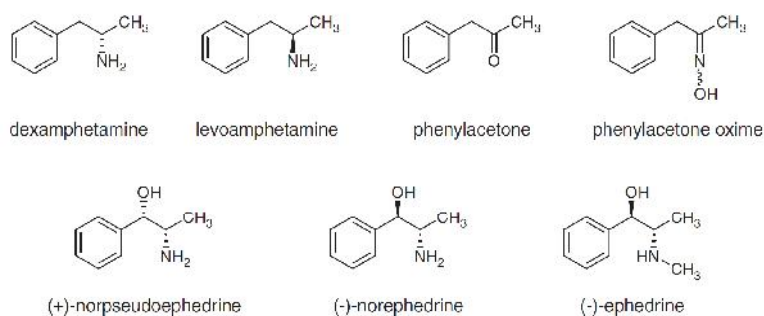


Figure 1. Structures of dexamphetamine, charged and neutral potential impurities as well as 1*R*,2*S*-(+)-ephedrine.

stereoisomeric purity of the drug by optical rotation, which is rather low and, therefore, prone to low sensitivity with regard to the detection of the *R*-enantiomer. Thus, several other techniques have been applied to the determination of the enantiomeric purity of dexamphetamine drug substance. These include enhancement of the optical rotation upon derivatization [13], NMR using europium shift reagents [14], complexation by heptakis-(2,3-di-*O*-acetyl)- β -CD [15, 16] or derivatization with 1*R*-(+)-myrtenal [17] as well as chromatographic techniques [18–20].

CE has been recognized as a suitable technique for the determination of the stereoisomeric purity of drugs [21–29] as well as the analysis of related substances [23, 24, 27, 30]. Moreover, it has been demonstrated that CE can be used to determine chiral impurities as well as (achiral) related substances simultaneously [31, 32]. Many publications have described the enantioseparation of amphetamine and other phenylalkylamines by CE, for example [33–39]. Thunhorst *et al.* compared CE and NMR for the determination of the enantiomeric composition of amphetamine upon complexation with heptakis-(2,3-di-*O*-acetyl)- β -CD [16] based on a fundamental study by Wedig and Holzgrabe on analyte complexation by this CD [40]. Moreover, an enantioselective CE assay for the simultaneous determination of the enantiomeric purity of dexamphetamine as well as the potential impurities 1*R*,2*S*-(+)-norephedrine and 1*S*,2*S*-(+)-norpseudoephedrine has been described recently [41]. However, as none of the charged impurities could be detected in commercial samples, it was hypothesized that the synthesis occurred starting from phenylacetone. Therefore, the aim of the present work was the development and validation of a stereoselective CE method for dexamphetamine allowing the simultaneous analysis of charged and neutral impurities. The structures of the investigated compounds are shown in Fig. 1.

2 Materials and methods

2.1 Chemicals

All chemicals were of analytical grade. Dexamphetamine sulfate and 1*R*,2*S*-(+)-norephedrine hydrochloride were from Fagron (Barsbüttel, Germany), racemic amphetamine sulfate, 1*R*,2*S*-(+)-ephedrine sulfate, phenylacetone and

sulfated β -CD (S- β -CD) were from Sigma-Aldrich Chemie (Deisenhofen, Germany), and 1*S*,2*S*-(+)-norpseudoephedrine hydrochloride was from Hünslers AG (Herrisau, Switzerland). Phenylacetone oxime was synthesized by reaction of phenylacetone with hydroxylamine and purification by vacuum distillation. Sulfobutylether(V)- β -CD (SBE(V)- β -CD, degree of substitution (DS) of 4.9, SBE(IV)- β -CD (DS 4) and SBE(VII)- β -CD (DS 7) were obtained from CyDex Pharmaceuticals (Lexxa, KS, USA). Sodium hydroxide solution was from Fisher Scientific (Schwerte, Germany) and phosphoric acid was from Carl Roth (Karlsruhe, Germany). All buffers and solutions were prepared in deionized, double-distilled water, filtered through a 0.2 μ m membrane filter, and degassed by sonication.

2.2 CE

A BioFocus 2000 instrument (Biorad, Munich, Germany) equipped with a diode array detector was employed. The separations were obtained in uncoated fused-silica capillaries 50 μ m id, 375 μ m od (BGB Analytik, Schloßblockelheim, Germany) with the total length of 40.2 cm and an effective length of 35 cm operated at 20°C. Detection was carried out at 200 nm. A new capillary was treated with 1 M sodium hydroxide for 10 min, 0.1 M sodium hydroxide for 20 min, 0.1 M phosphoric acid for 10 min, water for 10 min and finally the run buffer for 10 min. Between the injections, the capillary was flushed with 0.1 M sodium hydroxide for 3 min, water for 2 min and the background electrolyte for 5 min.

The optimized background electrolyte consisted of 50 mM sodium phosphate buffer prepared by adjusting 50 mM phosphoric acid to pH 3.0 with a 1 M sodium hydroxide solution. CDs were dissolved in the buffer solution after adjustment of the pH. The applied voltage was 10 kV (reversed polarity). Stock solutions (2 mg/mL) of phenylacetone and phenylacetone oxime were prepared in methanol. Stock solutions of other compounds including the internal standard were prepared at a concentration of 1.0 mg/mL in water. The stock solutions were subsequently diluted with water to the desired concentrations. Samples were introduced by hydrodynamic injection at 0.5 psi for 8 s.

Due to peak broadening after five to six injections the background electrolyte was changed after three injections.

2.3 Method validation

Method validation was performed according to the ICH guideline Q2(R1) [42] with regard to range, linearity, LOD and LOQ, and precision. Linearity was estimated by unweighted linear regression using the least squares method. LOD and LOQ were based on a signal-to-noise ratio of 3:1 and 10:1, respectively. Precision was determined at a low concentration (0.1%) and a high concentration (0.7 or 2.0% for levoamphetamine). Intraday precision was calculated from six replicate injections on the same day while interday precision was based on six injections on three consecutive days.

3 Results and discussion

3.1 Method development and optimization

The structures of the analytes are summarized in Fig. 1. The simultaneous analysis of charged and neutral compounds including enantiomer separations can be achieved in CE exploiting the carrier ability charged chiral selectors. Consequently, negatively charged CD derivatives have been employed for the enantioseparations of phenylalkylamines such as amphetamine, methamphetamine, ephedrine and others including randomly sulfated CDs [33, 35, 43, 44] and sulfobutylether-CDs [35, 45]. Thus, various negatively charged CDs, e.g. sulfated α -CD, sulfated β -CD, sulfated γ -CD, heptakis-(2,3-di-O-acetyl-6-O-sulfo)- β -CD, carboxymethyl- β -CD, carboxymethyl- γ -CD, sulfopropyl- β -CD, sulfopropyl- γ -CD and SBE(V)- β -CD, were investigated for their ability to separate the enantiomers of amphetamine and the neutral impurities phenylacetone and phenylacetone oxime in a 50.2/45 cm fused-silica capillary using 50 mM phosphate buffer, pH 2.5, and reversed polarity of the applied voltage. Sulfated β -CD and SBE(V)- β -CD provided baseline separations of the amphetamine enantiomers and phenylacetone and phenylacetone oxime. Sulfated β -CD displayed high resolution of the amphetamine enantiomers at a concentration of 20 mg/mL ($R_s > 3$) but neither phenylacetone nor the oxime could be detected within 60 min. Using a concentration of sulfated β -CD of 40 mg/mL the neutral analytes migrated at about 45 min but the peaks were rather broad. In contrast, a high concentration of 80 mg/mL of SBE(V)- β -CD was necessary for baseline resolution of the amphetamine enantiomers but phenylacetone and its oxime migrated before amphetamine. At concentrations of SBE(V)- β -CD of 70 mg/mL or below broad peaks and/or peak splitting was observed. Subsequently, a dual CD system comprising sulfated β -CD for the effective resolution of the amphetamine enantiomers and SBE(V)- β -CD for the separation of phenylacetone and

phenylacetone oxime was selected. Optimizing the CD concentration led to the composition of 25 mg/mL of sulfated β -CD and 80 mg/mL of SBE(V)- β -CD resulting in acceptable peak shape when injecting a solution of the analytes in the presence of 5 mg/mL dexamphetamine sulfate. This concentration of dexamphetamine was necessary in order to enable the determination of the impurities at the 0.1% level. Using a lower amount of the CDs in the background electrolyte led to peak shape distortion of levoamphetamine, which migrated immediately after dexamphetamine. Furthermore, the effective length of the capillary was shortened from 45 to 35 cm in order to reduce the analysis time. Using an applied voltage of -15 kV led to currents above 100 μ A, which led to poor peak resolution due to Joule heating. Thus, the voltage was reduced to -10 kV, which resulted in currents of about 75 μ A and acceptable peak shape. These conditions resulted in the separation of the enantiomers of amphetamine, the positively charged potential impurities 1S,2S-(+)-norpseudoephedrine and 1R,2S-(−)-norephedrine, the neutral compounds phenylacetone and phenylacetone oxime as well as 1R,2S-(−)-ephedrine (selected as internal standard) as shown in Fig. 2A.

During method development it was noted that phenylacetone oxime separated into two peaks when concentrations of SBE(V)- β -CD above 50 mg/mL were applied. Oximes can exist in two stereoisomers with E or Z configuration, which can be distinguished, among others, by NMR spectroscopy. ^1H -NMR spectroscopy of the synthetic sample revealed two signals at 3.78 and 3.53 ppm corresponding to the methylene groups of the Z and E isomer, respectively. The ratio was 26% Z-oxime and 74% E-oxime, which is in accordance with literature data [46]. Moreover, the peak area ratio of the two peaks was about 1:2.9, which matches the reported equilibrium. Therefore, the two peaks were assigned as phenylacetone Z-oxime and phenylacetone E-oxime. The partial overlap of the oximes could be overcome by raising the buffer pH from 2.5 to 3.0. Thus, the optimized conditions employed a 50 mM phosphate buffer, pH 3.0, containing 80 mg/mL of SBE(V)- β -CD and 25 mg/mL of sulfated β -CD as background electrolyte, a 50 μ m id fused-silica capillary with an effective length of 35 cm (total length 40.2 cm) at an applied voltage of -10 kV operated at 20°C. The separation of a solution of 5 mg/mL dexamphetamine containing about 3% of levoamphetamine and 1% of the impurities under these conditions is shown in Fig. 2B.

Sulfobutylether β -CD exists in qualities with different substitution patterns, which can affect stereoisomer separations [35, 47]. The composition of SBE- β -CD can be determined by CE and mass spectrometric techniques [48–51]. Thus, CDs with a different DS, i.e. SBE(IV)- β -CD (DS 4) and SBE(VII)- β -CD (DS 7), were investigated in comparison to SBE(V)- β -CD. Figure 3 summarizes electropherograms obtained with the three sulfobutylether β -CDs at a concentration of 80 and 20 mg/mL sulfated β -CD under otherwise identical conditions. A sample of 5 mg/mL dexamphetamine sulfate spiked with 1% of the potential

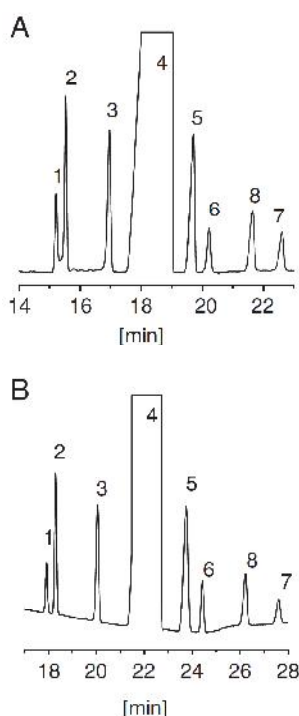


Figure 2. Effect of the pH of the background electrolyte on analyte separation of a sample of 5 mg/mL dexamphetamine sulfate containing about 3% levoamphetamine and spiked with 1% (50 µg/mL) of the impurities as well as 70 µg/mL ephedrine (IS). Background electrolyte: 50 mM sodium phosphate buffer (A) pH 2.5, (B) pH 3.0, containing 80 mg/mL SBE(V)-β-CD and 25 mg/mL S-β-CD. Experimental conditions: 40.2/35 cm, 50 µm id fused-silica capillary, 10 kV, 20 °C. 1, phenylacetone Z-oxime; 2, phenylacetone E-oxime; 3, phenylacetone; 4, dexamphetamine; 5, levoamphetamine; 6, 1S,2S-(+)-norpseudoephedrine; 7, 1R,2S-(-)-norephedrine; 8, 1R,2S-(-)-ephedrine.

impurities was analyzed. The DS affected peak shape and peak resolution, primarily the separation of the amphetamine enantiomers. While distorted peak of dexamphetamine was observed for SBE(IV)-β-CD (Fig. 3A), resolution between the amphetamine enantiomers was decreased using SBE(VII)-β-CD as chiral selector (Fig. 3B). In the case of SBE(V)-β-CD peak splitting of levoamphetamine was observed (Fig. 3C). As mentioned above, peak splitting could be avoided by increasing the concentration of sulfated β-CD to 25 mg/mL (Fig. 3D). Thus, adequate separation conditions for dexamphetamine and its potential impurities can be achieved by variation of the concentration of applied CDs. From the present results it may be concluded that a sample containing a high concentration of dexamphetamine and about 3–5% of levoamphetamine appears to be suitable for estimating the performance of the method as a system suitability test. Due to the fact that peaks with a large difference in concentration are utilized, the separation should be characterized by the peak-to-valley ratio. As can be seen in Fig. 3B the peaks of dexamphetamine and levoam-

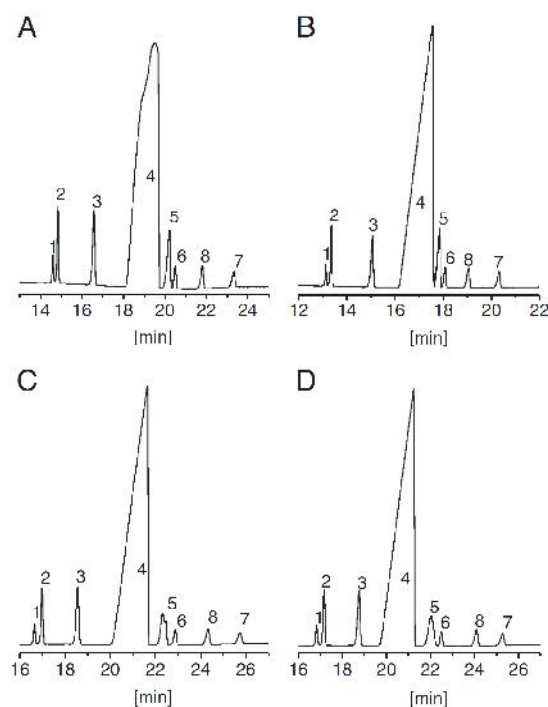


Figure 3. Effect of the DS of SBE-β-CD on peak shape and peak resolution. (A) SBE(IV)-β-CD, (B) SBE(VII)-β-CD, (C) SBE(V)-β-CD. A sample of 5 mg/mL dexamphetamine sulfate containing about 3% levoamphetamine and spiked with 1% (50 µg/mL) of the impurities as well as 70 µg/mL ephedrine. Background electrolyte: 50 mM phosphate buffer, pH 3.0, containing (A) 80 mg/mL SBE(IV)-β-CD and 20 mg/mL S-β-CD, (B) 80 mg/mL SBE(VII)-β-CD and 20 mg/mL S-β-CD, (C) 80 mg/mL SBE(V)-β-CD and 20 mg/mL S-β-CD, and (D) 80 mg/mL SBE(V)-β-CD and 25 mg/mL S-β-CD. For other experimental conditions and peak assignment see Fig. 2.

phetamine are not entirely separated but reliable quantitation is possible. The situation refers to a peak-to-valley ratio of 33. Thus, a peak-to-valley ratio of 30 could be defined as a system suitability test.

As discussed above, the concentration of the individual CDs and, subsequently, the composition of the chiral selectors affect peak shape and resolution (see also Fig. 3). Concentrations of SBE(V)-β-CD of 70 mg/mL or below affected the peak shape of dexamphetamine. Reducing the concentration of sulfated β-CD from 25 to 20 mg/mL led to peak deformation of levoamphetamine. Concentrations of sulfated β-CD of 30 mg/mL resulted in a partial overlap of the peaks of phenylacetone and dexamphetamine. Minor changes of the concentrations of both CDs in the range of ±1–2 mg/mL within the optimized conditions using 80 mg/mL SBE(V)-β-CD and 25 mg/mL sulfated β-CD did not show significant effects on peak resolution or peak shape of all components (data not shown). Nevertheless, careful preparation of the background electrolyte is recommended. As pointed out above, the

Table 1. Calibration data of levoamphetamine including potential charged and neutral impurities

Compound	Range(%) ^{a)}	Slope	Intercept	Correlation coefficient (r^2)	Residual sum of squares
Phenylacetone Z-oxime	0.05–1.0	1.9763	0.0043	0.9993	0.0009
Phenylacetone E-oxime	0.05–1.0	0.6876	−0.0005	0.9990	0.0013
Phenylacetone	0.05–1.0	0.6157	0.0093	0.9972	0.0037
1S,2S-(+)-Norpseudoephedrine	0.05–1.0	1.4059	−0.0190	0.9937	0.0082
1R,2S-(−)-Norephedrine	0.05–1.0	1.2549	0.0151	0.9982	0.0024
Levoamphetamine	0.05–5.0	1.0719	0.0053	0.9999	0.0025

a) Range corresponding to a concentration of 5 mg/mL dexamphetamine sulfate.

system can be easily adjusted by variations of the concentration of one of the CDs employed in order to achieve sufficient peak resolution and peak shape for the quantitation of all impurities.

3.2 Method validation

The optimized method was subsequently validated according to the ICH guideline Q2(R1) [42] with regard to linearity, range, LOD, LOQ as well as intraday and interday precision. Because enantiomerically pure dexamphetamine was not available, method validation was performed with racemic amphetamine. Phenylacetone, phenylacetone oxime, 1R,2S-(−)-norephedrine and 1S,2S-(+)-norpseudoephedrine were validated over the range of 2.5–50 µg/mL corresponding to 0.05–1.0% of the impurities based on a concentration of 5 mg/mL dexamphetamine sulfate. Due to the fact that concentrations of 3–4% levoamphetamine have been reported for dexamphetamine sulfate [40] the stereoisomer was validated in the range of 2.5–250 µg/mL corresponding to 0.05–5% of the compound. Ephedrine was used as internal standard at a concentration of 70 µg/mL in order to compensate for injection errors and minor fluctuations of the migration time. Calibration data obtained by unweighted linear regression are summarized in Table 1. Correlation coefficients of at least 0.993 were observed indicating sufficient linearity in the investigated concentration range. The 95% confidence intervals of the y-intercepts included zero for all compounds so that a systematic error can be excluded. The LOQ estimated at a signal-to-noise ratio of 10 was 0.05% (2.5 µg/mL) for all potential impurities. The LOD corresponding to a signal-to-noise ratio of 3 was 0.02% (1 µg/mL) for the charged analytes and 0.01% (0.5 µg/mL) for phenylacetone and its oxime. The intraday and interday precision was investigated at 0.1 and 0.7% for all impurities except for levoamphetamine, which was studied at 0.1 and 2.0%. The RSD values as summarized in Table 2 were always below 8% demonstrating sufficient repeatability of the method (Table 2). Figure 4 shows an electropherogram of a solution of 5 mg/mL of commercial dexamphetamine sulfate (containing about 3% levoamphetamine) spiked with the other impurities at the 0.1% level as well as 70 µg/mL of the internal standard ephedrine.

Table 2. Intraday and interday precision expressed as RSD values (intraday $n=6$; interday $n=3$)

Compound	Intraday		Interday	
	0.1%	0.7%/2.0% ^{a)}	0.1%	0.7%/2.0% ^{a)}
Phenylacetone Z-oxime	6.12	3.66	7.49	5.21
Phenylacetone E-oxime	5.77	2.61	5.32	5.73
Phenylacetone	2.01	2.72	5.18	5.13
1S,2S-(+)-Norpseudoephedrine	1.75	1.28	2.94	1.39
1R,2S-(−)-Norephedrine	1.40	0.85	3.18	1.54
Levoamphetamine	2.92	1.75	2.97	2.62

a) Levoamphetamine was determined at relative concentration of 2.0% while all other impurities were determined at a relative content of 0.7%.

3.3 Method application for drug analysis

The method was subsequently applied to the analysis of commercial samples of dexamphetamine sulfate. Samples were analyzed at a concentration of 5 mg/mL drug substance containing 70 µg/mL ephedrine as internal standard. The results are summarized in Table 3. In accordance with earlier results [41] neither 1R,2S-(−)-norephedrine nor 1S,2S-(+)-norpseudoephedrine could be detected. In contrast, peaks corresponding to phenylacetone and both phenylacetone oxime isomers were observed although these peaks were close to or below the LOD (Fig. 5). The European Pharmacopoeia defines that the LOQ corresponding to a signal-to-noise ratio of 10 is equal to or less than the disregard limit [52]. Thus, these impurities are below the disregard limit and would not be considered according to the regulations of the European Pharmacopoeia. However, their presence clearly demonstrates that the investigated batches of dexamphetamine sulfate were synthesized starting from phenylacetone via the oxime and subsequent enantioresolution by fractional crystallization. Levoamphetamine was present at concentrations between 3.2 and 3.7%. These data are in accordance with an earlier study, which reported concentrations of 2.8–4.0% levoamphetamine in dexamphetamine samples [41]. Using peak area normalization instead of the calibration data for the calculation of the levoamphetamine content

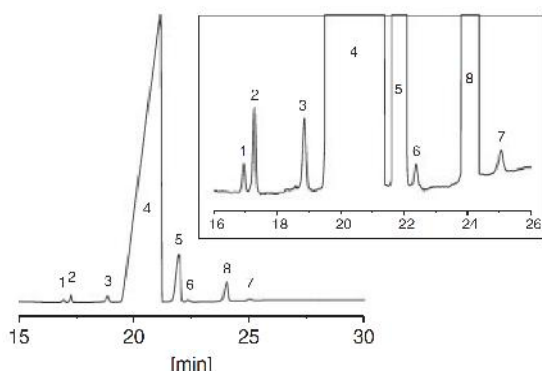


Figure 4. Electropherogram of a commercial sample of dexamphetamine sulfate containing about 3% levoamphetamine and spiked with other potential impurities at a concentration of 5 µg/mL (0.1% level) as well as 70 µg/mL ephedrine. Experimental conditions: 40.2/35 cm, 50 µm id fused-silica capillary, 50 mM sodium phosphate buffer, pH 3.0, containing 80 mg/mL SBE(V)-β-CD and 25 mg/mL S-β-CD, -10 kV, 20 °C. For peak assignment see Fig. 2.

Table 3. Impurity profile of commercial dexamphetamine sulfate samples (mean ± SD, *n*=3)

Compound	Sample 1	Sample 2	Sample 3	Sample 4
Phenylacetone Z-oxime	ND ^{a)}	LOD ^{b)}	LOD	LOD
Phenylacetone E-oxime	LOD	LOD	LOD	LOD
Phenylacetone	LOD	LOD	LOD	LOD
1 <i>S</i> ,2 <i>S</i> -(–)-Norpseudoephedrine	ND	ND	ND	ND
1 <i>R</i> ,2 <i>S</i> -(–)-Norpseudoephedrine	ND	ND	ND	ND
Levoamphetamine (calibration)	3.23 ± 0.02	3.70 ± 0.15	3.38 ± 0.09	3.36 ± 0.06
Levoamphetamine (normalization)	3.88 ± 0.06	4.25 ± 0.07	4.01 ± 0.11	3.47 ± 0.04

a) ND: not detectable.

b) LOD: detected at the LOD level.

values between 3.5 and 4.3% were found considering only the areas of the dexamphetamine and levoamphetamine peaks (Table 3). These data are generally higher but are in good agreement with the values obtained from the calibration data.

4 Concluding remarks

A CE method was developed and validated for the simultaneous determination of the stereoisomeric purity as well as related substances of dexamphetamine sulfate employing a dual CD system consisting of SBE(V)-β-CD and sulfated β-CD. The method allowed the quantitation of impurities at the 0.05% level. In accordance with an earlier study [41] 3.2 to 3.7% of levoamphetamine were found in

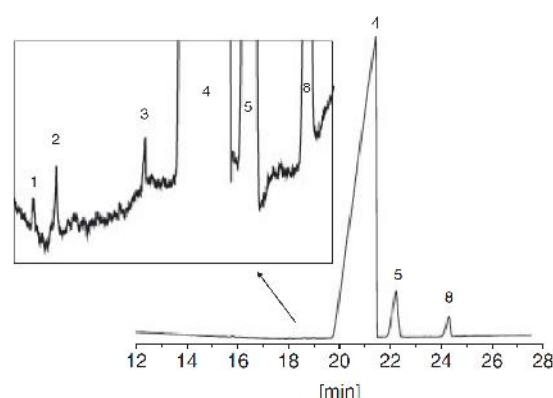


Figure 5. Electropherogram of a sample of commercial dexamphetamine sulfate analyzed at a concentration of 5 mg/mL. For experimental conditions see Fig. 4, for peak assignment see Fig. 2.

the investigated samples. Due to the fact that norephedrine and norpseudoephedrine could not be detected in contrast to the neutral impurities phenylacetone and phenylacetone oxime the synthetic origin of the investigated drug substance can be concluded. The present method also allowed the CE separation of the E and Z stereoisomers of phenylacetone oxime which, to the best of our knowledge, has not yet been reported.

The present study employed randomly substituted CDs, which may be prone to variation of the DS. This can affect separations as reported for sulfobutylether β-CD [35, 46] and also observed in the present study. However, the changes with regard to peak shape and peak resolution can be reversed by adjusting the concentration of the employed CDs. Therefore, a system suitability test using dexamphetamine and levoamphetamine with strongly differing concentrations as a critical pair should be employed when sulfobutylether β-CDs with a DS different from 5 are utilized.

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The authors have declared no conflict of interest.

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Manuscript III

Impurity profiling of dexamphetamine sulfate by cyclodextrin-modified microemulsion electrokinetic chromatography

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Research Article

Impurity profiling of dexamphetamine sulfate by cyclodextrin-modified microemulsion electrokinetic chromatography

A CD-modified microemulsion electrokinetic chromatography method has been developed and validated for dexamphetamine sulfate which allows the simultaneous determination of charged and uncharged impurities of the drug including the levorotary (*R*)-enantiomer. The optimized background electrolyte consisted of 1.5% w/w SDS, 0.5% w/w ethyl acetate, 3.5% w/w 1-butanol, 2.5% w/w 2-propanol and 92% w/w 50 mM sodium phosphate buffer, pH 3.0, containing 5.5% w/w sulfated β -CD. Separations were performed in a 50.2/40 cm, 50 μ m id fused silica capillary at a temperature of 20°C and an applied voltage of –14 kV. Carbamazepine was used as internal standard. The assay was validated in the range of 0.1–1.0% for the related substances and 0.1–5.0% for levoamphetamine based on a concentration of 3 mg/mL of dexamphetamine sulfate. The LOD of all analytes ranged between 0.05 and 0.2%. In commercial samples of dexamphetamine sulfate, levoamphetamine was found at concentrations between 3.2 and 3.8%, whereas none of the other impurities could be detected.

Keywords:

Dexamphetamine / Enantioseparation / Microemulsion electrokinetic chromatography
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1 Introduction

Dexamphetamine (dextroamphetamine, 2S-(+)-amphetamine, (*S*)-1-phenyl-2-propanamine, Fig. 1) is the dextrorotary (*S*)-enantiomer of the racemic drug amphetamine. Dexamphetamine is a psychostimulant drug which is used for the treatment of the attention-deficit/hyperactivity disorder of children [1, 2] and narcolepsy [3]. Dexamphetamine can be synthesized by various methods, for example, starting from chiral precursors such as 1*R*,2*S*-(–)-norephedrine or 1*S*,2*S*-(+)-norpseudoephedrine [4, 5]. Alternatively dexamphetamine can be obtained from racemic amphetamine synthesized from phenylacetone by Leuckart reaction or by reduction of phenylacetone oxime [6, 7] followed by fractional crystallization using L-(+)-tartaric acid [8]. Thus, charged as well as neutral starting materials or synthetic by-products as well as the enantiomer levoamphetamine (2*R*-(–)-amphetamine, Fig. 1) may be present as related substances in the drug.

The enantioseparation of amphetamine has been achieved by several analytical techniques including CE. Neutral and negatively charged CDs have been applied as chiral selectors in these studies, for example [9–13]. Recently, enantioselective capillary electrokinetic chromatographic assays employing CDs as chiral selectors have been developed for the simultaneous analysis of the stereoisomeric purity of dexamphetamine as well as the analysis of related substances. Using a phosphate buffer, pH 2.5, containing 10 mg/mL heptakis-(2,3-di-*O*-acetyl-6-*O*-sulfo)- β -CD levoamphetamine as well as the charged impurities 1*R*,2*S*-(–)-norephedrine or 1*S*,2*S*-(+)-norpseudoephedrine (Fig. 1) could be analyzed in the normal polarity mode [14]. A dual CD system comprising two negatively charged CDs, sulfolbutylether- β -CD and sulfated β -CD, allowed the determination of the uncharged impurities phenylacetone and phenylacetone oxime (Fig. 1) in addition to the charged impurities and levoamphetamine when reversing the polarity of the applied voltage [15].

Microemulsion electrokinetic chromatography (MEEKC) has proved to be a powerful technique for the analysis of charged and uncharged compounds including the stereoisomer analysis [16–21]. Stereoisomer separation in MEEKC can be achieved either by the use of chiral surfactants or a chiral oil core or by the addition of chiral selectors such as CDs to a “classical” microemulsion. The latter method is also called CD-modified MEEKC. This technique has been applied to the enantioseparation of esbiothrin [22], tropa alkaloids [23] or phenylalanine analogues including DOPA and methyl dopa

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Abbreviation: MEEKC, microemulsion electrokinetic chromatography

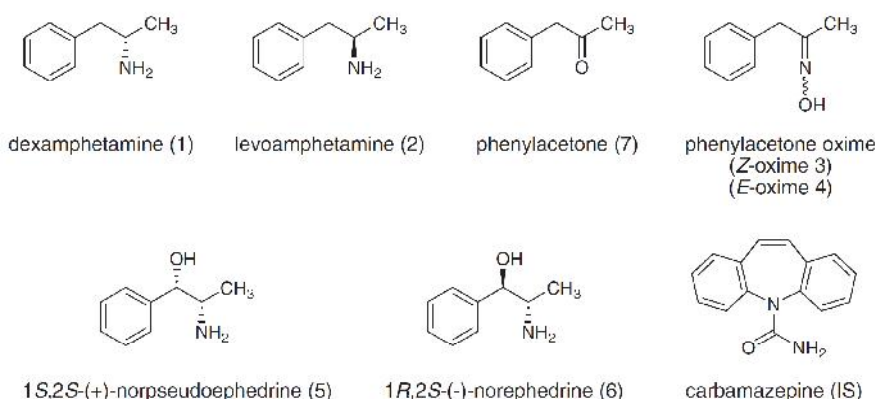


Figure 1. Structures of dexamphetamine and potential impurities as well as the internal standard carbamazepine.

[24]. Moreover, the analysis of impurities of oxybutynin has been reported which included the enantioseparation of one impurity [25]. However, to the best of our knowledge, the simultaneous application of MEEKC to the determination of achiral impurities of drugs and the analysis of enantiomeric impurities has not been reported to date. Thus, this study was conducted in order to evaluate CD-modified MEEKC for the impurity profiling of drugs including the determination of the stereoisomeric purity using dexamphetamine sulfate as an example. From an analytical standpoint, the compounds comprised charged and uncharged analytes, a pair of enantiomers as well as another pair of geometrical isomers, i.e. *E*- and *Z*-isomers.

2 Materials and methods

2.1 Chemicals

Dexamphetamine sulfate and 1R,2S-(-)-norephedrine hydrochloride were from Fagron GmbH (Barsbüttel, Germany). Racemic amphetamine sulfate, 1R,2S-(-)-ephedrine sulfate, carbamazepine, phenylacetone and sulfated β -CD were from Sigma-Aldrich Chemie (Deisenhofen, Germany). SDS was from Merck (Darmstadt, Germany) and 1S,2S-(+)-norpseudoephedrine hydrochloride was from Hanseler (Herisau, Switzerland). Phenylacetone oxime was synthesized by the reaction of phenylacetone with hydroxylamine and purification by vacuum distillation. Phosphoric acid was from Carl Roth (Karlsruhe, Germany). All other chemicals were of analytical grade. All buffers and solutions were prepared in deionized, double-distilled water, filtered through a 0.2 μ m membrane filter, and degassed by sonication before use.

2.2 Instrumentation

The experiments were carried out on a Beckman Coulter P/ACE System MDQ (Fullerton, CA, USA) equipped with a DAD detector set at 200 nm. The separations were

performed in uncoated fused silica capillaries 50 μ m id, 375 μ m od (BGB Analytik, Schlobockelheim, Germany) with a total length of 50.2 cm and an effective length of 40 cm at a temperature of 20 $^{\circ}$ C. A new capillary was treated with 1.0 M sodium hydroxide for 20 min, 0.1 M hydrochloric acid for 10 min, water for 10 min and the background electrolyte for 10 min. Between the injections, the capillary was flushed with 0.1 M sodium hydroxide for 3 min, water for 2 min, methanol for 3 min, 0.1 M phosphoric acid for 2 min, water for 2 min and the buffered microemulsion for 8 min, respectively. Samples were introduced by hydrodynamic injection at 0.5 psi for 4 s and separated at an applied voltage of -14 kV (reversed polarity). The background electrolyte was changed every three injections due to increased migration times and peak broadening that were observed in the case of prolonged use.

2.3 Preparation of sample solutions and microemulsion background electrolyte

Stock solutions (2.0 mg/mL) of phenylacetone and phenylacetone oxime as well as the internal standard carbamazepine (1.0 mg/mL) were prepared in methanol. Stock solutions of the other compounds were prepared at a concentration of 1.0 mg/mL in water. The sample solutions were subsequently diluted with water to the desired concentrations. Fifty mM sodium phosphate buffer was prepared by adjusting 50 mM phosphoric acid to pH 3.0 with a 1.0 M sodium hydroxide solution. The optimized background electrolyte consisted of 1.5% w/w SDS, 0.5% w/w ethyl acetate, 3.5% w/w 1-butanol, 2.5% w/w 2-propanol, 5.5% w/w sulfated β -CD and 92% w/w 50 mM phosphate buffer, pH 3.0. The microemulsion was prepared in the following way: sulfated β -CD and phosphate buffer were weighed and sonicated for 5 min (power 60 W with a frequency of 45 kHz). This solution was transferred into a flask containing the appropriately weighed amounts of SDS, ethyl acetate and 1-butanol. The resulting mixture was sonicated for 20 min. Subsequently, the appropriate amount of 2-propanol was added.

2.4 Method validation

The optimized method was validated according to the ICH guideline Q2(R1) [26] (<http://www.ich.org>) with regard to range, linearity, LOD and LOQ, and precision. Linearity was estimated by unweighted linear regression using the least-squares method. The LOD and LOQ were based on a signal-to-noise ratio of 3:1 and 10:1, respectively. Precision was determined at a low concentration (0.2 or 0.5% for the Z-isomer) and a high concentration (0.8%) for the impurities except for levoamphetamine where precision was determined at 1 and 3%. Intraday precision was calculated from six replicate injections on the same day, whereas interday precision was based on the six injections on three consecutive days.

3 Results and discussion

3.1 Method development and optimization

The structures of the analytes are shown in Fig. 1. Phenylacetone oxime exists as two stereoisomers, the *E*-isomer and the *Z*-isomer, at a ratio of about 3:1 [15, 27]. Initially MEKC was evaluated for the simultaneous separation of charged and neutral related substances and amphetamine using background electrolytes with pH values of 9.5 and 3.0 containing 3.5% w/w SDS and various amounts of organic solvents such as 1-butanol, 2-propanol, acetonitrile or methanol. However, none of the tested conditions resulted in acceptable peak shape, baseline noise or (achiral) separation of all analytes. Thus, MEEKC was further considered. Borst and Holzgrabe recently published a CD-modified MEEKC assay for the enantioseparation of phenylalanine derivatives [24]. The microemulsion consisted of 20–40 mM sodium phosphate buffer, pH 2.0–2.5, ethyl acetate, SDS, 1-butanol, 2-propanol and sulfated β -CD. All analytes were resolved by variation of the buffer pH, composition of the microemulsion and CD concentration. Thus, a microemulsion containing these components was selected and subsequently optimized for the simultaneous separation of dexamphetamine and the related substances including levoamphetamine. A solution of 2 mg/mL dexamphetamine sulfate containing about 3% levoamphetamine and 1% of the impurities was used for method development. The initial sample also contained 1*R*,2*S*-(–)-ephedrine which had been used as internal standard in the enantioselective capillary electrokinetic chromatographic assays [14, 15].

Figure 2A shows the separation of the analytes using a microemulsion composed of 1.4% w/w SDS, 1.0% w/w ethyl acetate, 4.0% w/w 1-butanol, 2.8% w/w 2-propanol, 90.8% w/w 50 mM phosphate buffer, pH 3.0, containing 40% w/w sulfated β -CD under reversed polarity of the applied voltage. Although the enantiomers of amphetamine were well resolved, the intended internal standard 1*R*,2*S*-(–)-ephedrine comigrated with 1*S*,2*S*-(+)-norpseudoephedrine and the *Z*-stereoisomer of phenylacetone oxime was not completely

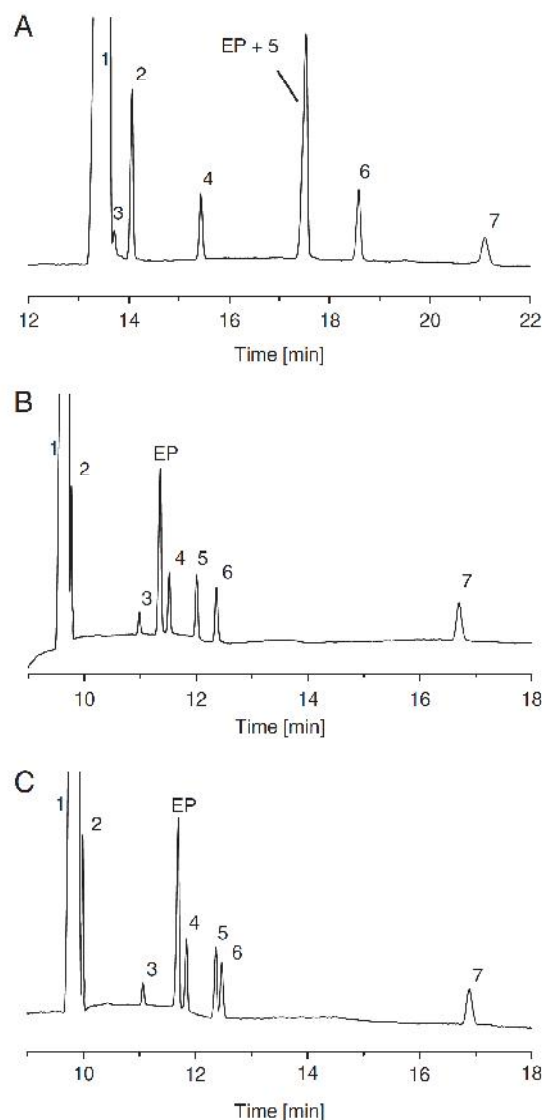


Figure 2. Effect of the composition of the microemulsion on analyte separation. Background electrolyte (A) 1.4% w/w SDS, 1.0% w/w ethyl acetate, 4.0% w/w 1-butanol, 2.8% w/w 2-propanol, 90.8% phosphate buffer, pH 3.0, containing 4.0% w/w sulfated β -CD; (B) 1.5% w/w SDS, 0.5% w/w ethyl acetate, 3.0% w/w 1-butanol, 2.0% w/w 2-propanol, 93.0% w/w phosphate buffer, pH 3.0, containing 4.0% w/w sulfated β -CD and (C) 1.5% w/w SDS, 1.0% w/w ethyl acetate, 3.0% w/w 1-butanol, 2.0% w/w 2-propanol, 92.5% w/w phosphate buffer, pH 3.0, containing 4.0% w/w sulfated β -CD. Other experimental conditions: 50.2/40 cm, 50 μ m id fused silica capillary, 20 °C, 15 kV; sample: 2 mg/mL dexamphetamine sulfate containing about 3% levoamphetamine and 1% of the other impurities as well as 70 μ g/mL 1*R*,2*S*-(–)-ephedrine (EP). For peak assignment, see Fig. 1.

separated from dexamphetamine. Increasing the concentration of SDS to 1.5% w/w did not significantly affect the separation, whereas a further increase to 2.0% w/w resulted in

a loss of the enantioresolution of amphetamine and comigration of 1*R*,2*S*-(–)-ephedrine, 1*S*,2*S*-(+)-norpseudoephedrine and phenylacetone *E*-oxime (data not shown). Thus, a concentration of 1.5% w/w SDS was selected for further studies. On the contrary, the content of ethyl acetate as the oil phase had a significant effect on the separation of the analytes. Reduction of the ethyl acetate content to 0.5% w/w with a concomitant reduction of the co-surfactant 1-butanol to 3.0% w/w and the organic modifier 2-propanol to 2.0% w/w resulted in at least partial separation of all analytes (Fig. 2B). Only incomplete enantioseparation of amphetamine was observed. Increasing ethyl acetate to 1.0% w/w improved the enantioseparation but led to partial overlap of 1*S*,2*S*-(+)-norpseudoephedrine and 1*R*,2*S*-(–)-norephedrine (Fig. 2C). Thus, a concentration of 0.5% w/w ethyl acetate was selected for further optimization of the CD concentration in order to improve the enantioseparation of dexamphetamine and levoamphetamine. Increasing sulfated β -CD to 5.5% w/w resulted in baseline separation of the enantiomers. However, upon a further increase of sulfated β -CD above 6.0% w/w an unstable baseline as well as partial overlap of phenylacetone *Z*-oxime and dexamphetamine was observed. Because partial comigration of phenylacetone *E*-oxime and 1*R*,2*S*-(–)-ephedrine was observed at concentrations of sulfated β -CD above 5%, 1*R*,2*S*-(–)-ephedrine was abandoned as internal standard. From a screening of amino acids and their derivatives as well as structurally not related compounds, carbamazepine was selected due to the peak shape and short migration time. Subsequently, the concentration of sulfated β -CD was set at 5.5% in order to avoid currents above 100 μ A. The final fine tuning of the microemulsion was performed by variation of the contents of 1-butanol between 3.0 and 4.0% w/w and of 2-propanol in the range between 2.0 and 3.0% w/w. The best separation of the analytes was achieved using 3.5% w/w 1-butanol and 2.5% w/w 2-propanol. Reduction of the buffer concentration to 20 mM resulted in peak broadening, whereas an increase of the concentration to 100 mM led to currents above 10 μ A. Upon decreasing the buffer pH to 2.0, reduced peak heights of the stereoisomers of phenylacetone oxime were observed, whereas the other compounds were not affected. Consequently, the final method employed a background electrolyte containing 1.5% w/w SDS, 0.5% w/w ethyl acetate, 3.5% w/w 1-butanol, 2.5% w/w 2-propanol and 92% w/w 50 mM phosphate buffer, pH 3.0, containing 5.5% w/w sulfated β -CD. Analyses were carried out at 20°C and in a 50 μ m id fused silica capillary with an effective length of 40 cm. At an applied voltage of –14 kV currents ranged between 73 and 78 μ A. The separation of a sample of 3 mg/mL dexamphetamine sulfate containing about 3% of levoamphetamine and 1% of the impurities as well as 50 μ g/mL of the internal standard is shown in Fig. 3A.

As observed during method development, preparation of the microemulsion affected the separation between dexamphetamine and levoamphetamine as well as between phenylacetone *E*-oxime and 1*S*,2*S*-(+)-norpseudoephedrine depending on the addition of sulfated β -CD either before or after the preparation of the microemulsion as shown in Fig. 3. The

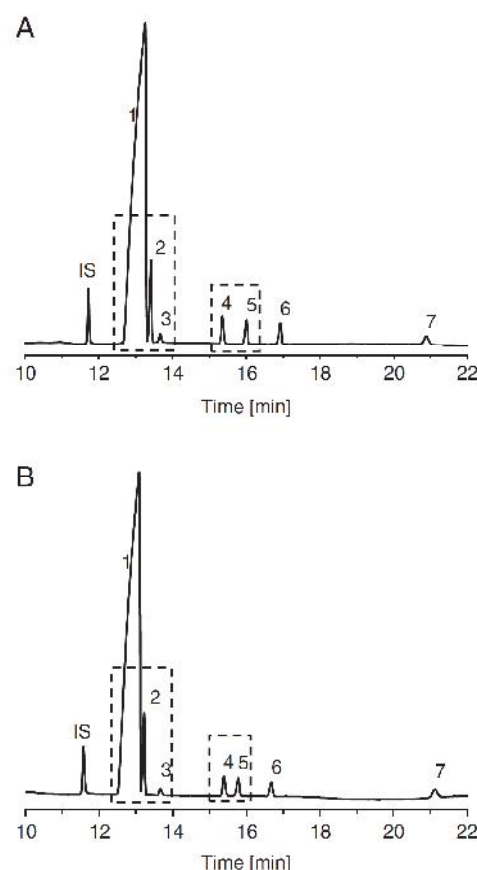


Figure 3. Effect of preparation of the background electrolyte on analyte separation. (A) Addition of sulfated β -CD to the phosphate buffer prior to the preparation of the microemulsion by sonication and (B) addition of sulfated β -CD after preparation of the microemulsion. For further details, see text. Experimental conditions: 50.2/40 cm, 50 μ m id fused silica capillary, 20°C, –14 kV; background electrolyte: 1.5% w/w SDS, 0.5% w/w ethyl acetate, 3.5% w/w 1-butanol, 2.5% w/w 2-propanol 92% w/w 50 mM sodium phosphate buffer, pH 3.0, containing 5.5% w/w sulfated β -CD; sample: 3 mg/mL dexamphetamine sulfate containing about 3% levoamphetamine and 30 μ g/mL of the other impurities as well as 50 μ g/mL carbamazepine as internal standard (IS). For peak assignment, see Fig. 1.

electropherogram in Fig. 3A shows the separation of the analytes when sulfated β -CD was dissolved in the phosphate buffer prior to preparation of the microemulsion by sonication of the aqueous phase with SDS, ethyl acetate and 1-butanol. Figure 3B shows the electropherogram obtained from a microemulsion with identical composition but addition of sulfated β -CD after formation of the microemulsion by sonication of phosphate buffer, SDS, ethyl acetate and 1-butanol. In both cases, the organic modifier 2-propanol was added last. There is no apparent explanation for this phenomenon but it emphasizes that the preparation of a microemulsion in MEEKC may affect a given separation and, therefore, has to be given consideration. Because the resolution between

dexamphetamine and levoamphetamine was slightly better when sulfated β -CD was added to the phosphate buffer prior to the preparation of the microemulsion, this background electrolyte was used for validation and sample analysis. In order to estimate the performance of the method a system, suitability test may be performed utilizing a sample of dexamphetamine at a concentration of 3 mg/mL containing 3–5% levoamphetamine. The enantioseparation of amphetamine is the most critical pair of compounds in the present assay. As peaks with a large difference in concentration are used, the separation should be characterized by the peak-to-valley ratio. The separation shown in Fig. 3A refers to a peak-to-valley ratio of about 45.

3.2 Method validation

The optimized method was subsequently validated according to the ICH guideline Q2(R1) [26] with regard to linearity, range, LOD, LOQ as well as intraday and interday precision. Because enantiomerically pure dexamphetamine was not available, method validation was performed with racemic amphetamine. Levoamphetamine contents of 3–4% have been reported for dexamphetamine sulfate [14, 15]. Therefore, the compound was validated in the range of 3–150 μ g/mL corresponding to 0.1–5% based on a concentration of 3 mg/mL dexamphetamine sulfate. The other potential impurities were validated in the range of 3–30 μ g/mL (0.1–1%) except for phenylacetone Z-oxime which was calibrated between 15 and 30 μ g/mL (0.5–1%). Carbamaz-

pine was used as internal standard at a concentration of 50 μ g/mL in order to compensate for injection errors and minor fluctuations of the migration time. Calibration data obtained by unweighted linear regression are summarized in Table 1. Correlation coefficients of at least 0.9938 were observed indicating sufficient linearity in the investigated concentration range. The 95% confidence intervals of the y-intercepts included zero for all compounds so that a systematic error can be excluded. The LOQ estimated at a signal-to-noise ratio of 10 was 0.1% (3 μ g/mL) for all potential impurities except for the Z-oxime with a LOQ of 0.5% (15 μ g/mL). The LOD corresponding to a signal-to-noise ratio of 3 was 0.05% (1.5 μ g/mL) for the compounds, whereas the LOD of the Z-oxime was 0.2% (6 μ g/mL). The intraday and interday precision was investigated at 0.2% (0.5% for the Z-oxime) and 0.8% for the related substances. Levoamphetamine was studied at 1 and 3%. The RSD values as summarized in Table 2 were always below 8.2%, demonstrating sufficient repeatability of the method.

3.3 Method application

Four commercial samples of dexamphetamine sulfate were subsequently analyzed using the validated method. Levoamphetamine was detected in the dexamphetamine samples at concentrations between 3.22 ± 0.10 and $3.82 \pm 0.07\%$ ($n = 3$). These data are in accordance with earlier CE studies reporting a content of levoamphetamine between

Table 1. Calibration data of levoamphetamine including potential charged and neutral impurities

Compound	Range (%) ^{a)}	Slope	Intercept	Correlation coefficient (r^2)	Residual sum of squares	LOD (μ g/mL)	LOQ (μ g/mL)
Levoamphetamine	0.1–5.0	1.9721	0.2289	0.9956	0.1503	1.5	3
Phenylacetone Z-oxime	0.5–1.0	5.7537	0.0829	0.9986	0.0006	6	15
Phenylacetone E-oxime	0.1–1.0	2.3170	0.0532	0.9964	0.0044	1.5	3
1 <i>S</i> ,2 <i>S</i> -(+)-norpseudoephedrine	0.1–1.0	2.3129	0.0647	0.9974	0.0031	1.5	3
1 <i>R</i> ,2 <i>S</i> -(−)-norephedrine	0.1–1.0	2.3271	0.0788	0.9957	0.0052	1.5	3
Phenylacetone	0.1–1.0	2.5942	0.0587	0.9938	0.0075	1.5	3

a) Range corresponding to a concentration of 3 mg/mL dexamphetamine sulfate.

Table 2. Intraday and interday precision expressed as RSD values (intraday $n = 6$; interday $n = 3$)

Compound	Intraday		Interday	
	0.2%/1% ^{a)}	0.8%/3% ^{a)}	0.2%/1% ^{c)}	0.8%/3% ^{a)}
Levoamphetamine	4.87	3.14	5.64	5.91
Phenylacetone Z-oxime	7.12 ^{b)}	6.88	5.12 ^{b)}	7.70
Phenylacetone E-oxime	8.13	5.30	6.78	6.40
1 <i>S</i> ,2 <i>S</i> -(+)-norpseudoephedrine	5.26	3.91	2.56	2.85
1 <i>R</i> ,2 <i>S</i> -(−)-norephedrine	7.50	4.09	5.84	3.73
Phenylacetone	4.22	5.03	4.51	5.17

a) Levoamphetamine was determined at relative concentration of 1.0 and 3.0% for a low and high concentration, respectively.

b) The precision at the low concentration of phenylacetone Z-oxime was performed at the LOQ of 0.5%.

2.79 ± 0.23 and $3.97 \pm 0.15\%$ [14] or 3.23 ± 0.02 and $3.70 \pm 0.15\%$ [15] using a single CD or a dual CD system, respectively. Applying peak area normalization for the calculation of the levoamphetamine, content values between 4.07 ± 0.03 and $4.66 \pm 0.03\%$ were found which are generally higher than the values obtained from the calibration data. In accordance with the earlier studies [14, 15] neither 1R,2S-(–)-norephedrine nor 1S,2S-(+)-norpseudoephedrine could be detected. However, although phenylacetone and the stereoisomers of phenylacetone oxime could be detected at the LOD level in the EKC assay [15], none of the compounds could be detected in the MEEKC assay. This can be attributed to the lower LODs of about 0.01–0.03% achieved with the EKC assay with the dual CD system due to the lower baseline noise obtained under these conditions.

4 Concluding remarks

A CD-modified MEEKC assay has been developed and validated that allows the simultaneous analysis of charged and uncharged impurities of dexamphetamine including the enantiomer levoamphetamine. The optimized background electrolyte consisted of 1.5% w/w SDS, 0.5% w/w ethyl acetate, 3.5% w/w 1-butanol, 2.5% w/w 2-propanol and 92% w/w 50 mM sodium phosphate buffer, pH 3.0, containing 5.5% w/w sulfated β -CD as a chiral selector. The fact that the time point of the addition of the CD during preparation of the microemulsion affected the resolution between the peaks of some compounds demonstrates that the way of the preparation of complex background electrolytes should be included in method development and validation.

Except for the Z-stereoisomer of phenylacetone oxime, the method allowed the quantitation of the impurities at the 0.1% level. In accordance with earlier EKC studies [14, 15], 3.2–3.8% of levoamphetamine were found in the investigated samples. However, especially the EKC assay employing a dual CD system proved to be more sensitive with LOQs of 0.05% of all analytes due to the lower background noise compared with MEEKC. Nevertheless, this study demonstrated for the first time that CD-modified MEEKC can be a powerful analytical technique for the simultaneous analysis of charged and uncharged impurities of compounds including stereoisomers. To the best of our knowledge, the determination of the stereoisomeric purity of a drug by MEEKC has not been published previously. Moreover, in the present case, a mixture containing a diversity of analytes, i.e. charged and uncharged related substances, enantiomers as well as E- and Z-geometrical isomers, could be separated simultaneously in a single run.

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The authors have declared no conflict of interest.

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Manuscript IV

Determination of related substances of levodopa including the *R*-enantiomer by CE

Sudaporn Wongwan, Manuela Hammitzsch-Wiedemann, Gerhard K.E. Scriba, *Electrophoresis* 2009, 30, 3891–3896.

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Research Article

Determination of related substances of levodopa including the *R*-enantiomer by CE

A CE assay for the simultaneous determination of the impurities of levodopa listed in the European Pharmacopoeia including the (*R*)-enantiomer was developed and validated. The analysis was performed in a fused-silica capillary employing sulfated β -cyclodextrin as chiral selector at an applied voltage of 20 kV and a temperature of 18°C. The optimized background electrolyte consisted of 0.1 M sodium phosphate buffer, pH 2.0, containing 6 mg/mL sulfated β -cyclodextrin. L-phenylalanine was used as the internal standard. The assay was validated in the range of 0.1–1.0% for the impurities at a concentration of levodopa of 2 mg/mL. The effect of different batches of sulfated β -cyclodextrin was investigated using levodopa and L-tyrosine as critical pair. The method was applied to determine the purity of several samples of levodopa including the chemical reference substance of the European Pharmacopoeia.

Keywords:

CE / Cyclodextrin / Enantiomer separation / Impurity profiling / Levodopa
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1 Introduction

Levodopa is a chiral drug used in the treatment of Parkinson's disease which is related to the depletion of the neurotransmitter dopamine in the brain. Chemically, levodopa is the (*S*)-enantiomer of 3,4-dihydroxyphenylalanine (DOPA, Fig. 1). Only this enantiomer is converted by the enzyme L-amino acid decarboxylase to dopamine in the brain while the (*R*)-enantiomer of DOPA (3,4-dihydroxy-D-phenylalanine, D-DOPA) is not converted and may cause side effects [1]. Moreover, as a synthetic drug levodopa may contain impurities related to the synthesis or degradation products which may be toxic. For example, 3,4,6-trihydroxyphenylalanine (6-OH-DOPA) is a neurotoxin [2]. Thus, the quality of the drug has to be assured for a safe therapy.

Levodopa is described in monographs in the European Pharmacopoeia [3] and the United States Pharmacopoeia [4] both including tests for the determination of related substances and of the enantiomeric purity. The related substances specified by the pharmacopoeias include L-Tyr, 3-methoxy-L-tyrosine (3-OMe-Tyr), and 6-OH-DOPA (Fig. 1). They are monitored by comparable HPLC assays in

both pharmacopoeias. In contrast, the stereochemical purity of levodopa is determined by optical rotation by the United States Pharmacopoeia based on the increased optical rotation of levodopa in the presence of methenamine [5] while the European Pharmacopoeia uses enantioselective HPLC based on a chiral ligand exchange method published by Doležalová and Tkaczyková [6]. An LOD of D-DOPA of 0.04% of the major enantiomer levodopa was reported, which is more sensitive than the measurement of the optical rotation of the sample. However, the method is somewhat hampered by the fact that negative peaks are observed between the peaks of levodopa and D-DOPA, which may make reproducible peak integration difficult. Other HPLC assays for the resolution of the enantiomers of DOPA have been reported employing various chiral stationary phases [7–11].

With regard to CE, several studies have been published for the enantioseparation of DOPA using chiral selectors such as CDs [12–16] including their use in MEKC [17] and MEEKC [18], the chiral crown ether (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid [19, 20], and chiral ligand exchange [21, 22]. Several of these methods were also validated and proved to be suitable for the determination of the enantiomeric purity of levodopa with LOD values of D-DOPA in the range of 0.02–0.1% of the major enantiomer [12–14, 17, 19]. In addition, the separation of levodopa and the related substances 3-OMe-Tyr and 6-OH-DOPA by CE has been reported [23]. However, no study has been published for the simultaneous determination of both, the chiral impurity as well as the related substances of levodopa. Theoretically, such a method could be used instead of the two individual tests of related substances and enantiomeric purity in a pharmacopoeia.

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Abbreviations: DOPA, 3,4-dihydroxyphenylalanine; D-DOPA, (3,4-dihydroxy)-D-phenylalanine; 3-OMe-Tyr, 3-methoxy-L-tyrosine; 6-OH-DOPA, (3,4,6-trihydroxy)-DL-phenylalanine; Phe, L-phenylalanine; Tyr, L-tyrosine

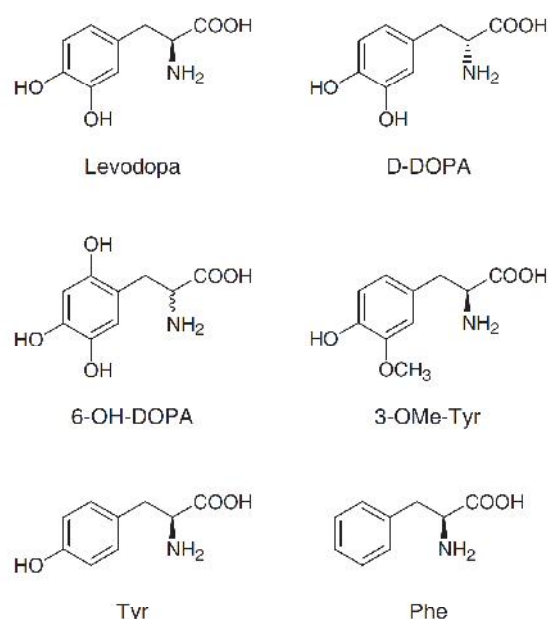


Figure 1. Structures of levodopa, D-DOPA and related substances as well as the internal standard L-Phe.

2 Materials and methods

2.1 Chemicals

Levodopa was obtained from Acros Organics (Geel, Belgium), levodopa chemical reference substance was from the European Directorate of the Quality of Medicines & Healthcare (Strasbourg, France). Racemic 3,4-dihydroxyphenyl-DL-alanine (DOPA) and L-phenylalanine (Phe) were purchased from Fluka BioChemika (Buchs, Switzerland), L-tyrosine (Tyr) from E. Merck (Darmstadt, Germany), and racemic 6-hydroxy-DL-DOPA (6-OH-DOPA) and 3-OMe-Tyr from Sigma-Aldrich Chemie (Steinheim, Germany). Different batches of sulfated β -CD (as sodium salt) were purchased from Fluka BioChemika or Sigma-Aldrich Chemie. All other chemicals were of analytical grade. Buffers and sample solutions were prepared on a daily basis in double-distilled, deionized water, filtered (0.2 μ m) and degassed by sonication.

2.2 CE

The experiments were carried out using a HP³¹⁰ CE system (Hewlett-Packard, Waldbronn, Germany) equipped with a diode-array detector. Instrument control, data acquisition, and data analysis were performed by the HP³¹⁰ CE ChemStation software. The detector wavelength was set to 200 nm with a bandwidth of 10 nm. Analyses were performed at an applied voltage of 20 kV, the temperature of the cartridge was set to 18 °C. Fused-silica capillaries of

50 μ m id, 375 μ m od were purchased from BGB Analytik (Schloßböckelheim, Germany). The total length was 45.2 cm, the effective length was 35 cm. A new capillary was conditioned by rinsing at a pressure of 50 mbar with 0.1 M sodium hydroxide solution for 10 min, water for 3 min, 0.1 M hydrochloric acid for 20 min, water for 10 min, and the running buffer for 10 min. Between runs the capillary was washed with 0.1 M sodium hydroxide solution for 3 min, 0.1 M phosphoric acid for 2 min, water for 2 min, and running buffer for 5 min.

0.1 M sodium phosphate buffer was prepared by adjusting 0.1 M phosphoric acid to pH 2.0 by the addition of 0.1 M sodium hydroxide solution. The CDs were added prior to the adjustment of the pH. All analytes were dissolved in 0.1 M hydrochloric acid and stored at –20 °C if not analyzed immediately. Samples were injected at a pressure of 50 mbar for 4 s.

3 Results and discussion

3.1 Method development and optimization

The structures of levodopa, D-DOPA and the related substances are summarized in Fig. 1. The enantiomers of DOPA have been separated in many CE studies employing chiral selectors including CDs, (+)-(18-crown-6)-2,3,11,12-tetracarboxylic acid or ligand exchange [12–22]. Systematic studies on the enantioseparations of DOPA by CDs have been performed by Saraç *et al.* [12] and Ha *et al.* [14] demonstrating that especially negatively charged CDs such as sulfated β -CD, sulfobutylether- β -CD or heptakis(2,3-di-O-acetyl-6-O-sulfo)- β -CD are effective selectors for the separation of the enantiomers of the drug. Due to its wide commercial availability and low price, sulfated β -CD was selected in the present study despite the fact that this CD is a randomly substituted CD derivative which may hamper the reproducibility of the assay depending on the commercial source and batch of the selector used.

The CE analysis of levodopa, 3-OMe-Tyr, and 6-OH-DOPA as related substances has been achieved in a 0.1 M sodium phosphate buffer, pH 8.5 [23], while acidic background electrolytes with a pH of 2.45–2.5 were used for the enantioseparations of DOPA using negatively charged CD derivatives [12–14, 16]. Under these conditions the analytes are positively charged while the selector is negatively charged which maximizes their interaction. Starting with a sodium phosphate buffer prepared from sodium phosphate containing 2 mg/mL sulfated β -CD with normal polarity of the applied voltage successive variation of the buffer pH between 2.0 and 3.0 as well as buffer molarity between 20 mM and 0.2 M was evaluated in a capillary with an effective length of 40 cm and an applied voltage of 20 kV. At pH 3.0 the analytes migrated very slowly due to their partial deprotonation at this pH while sharp peaks with short migration times were observed

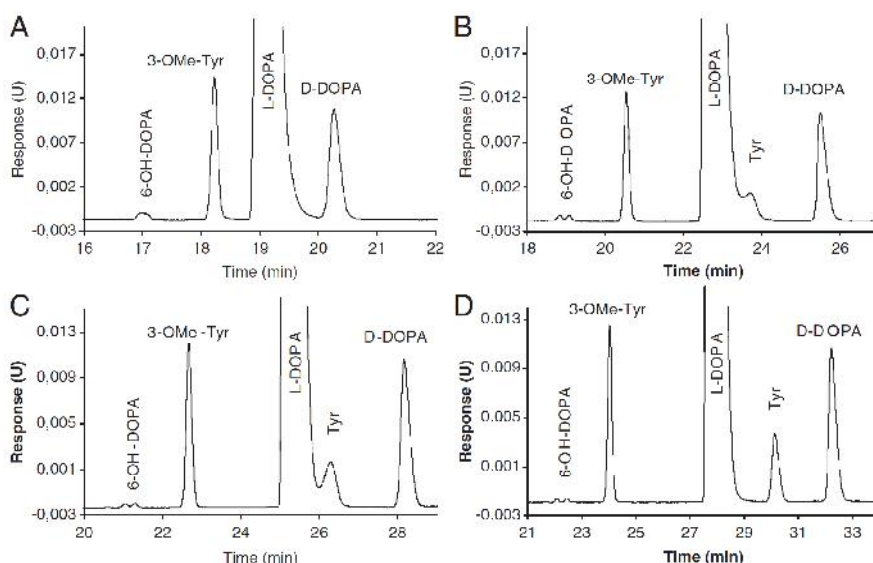


Figure 2. Effect of the concentration of sulfated β -CD on the separation of levodopa and its specified impurities. Experimental conditions: 0.12 M phosphate buffer, pH 2.0, containing (A) 2 mg/mL sulfated β -CD, (B) 4 mg/mL sulfated β -CD, (C) 6 mg/mL sulfated β -CD, and (D) 8 mg/mL sulfated β -CD; 50.2/40 cm fused-silica capillary, 20 kV, 18 °C. Sample concentration: 2 mg/mL levodopa containing 1% of 3-OMe-Tyr and D-DOPA, 0.5% of Tyr and 0.1% of 6-OH-DOPA.

at pH 2.0. With regard to buffer molarity, a 0.12 M buffer, pH 2.0 provided a good compromise between peak shape and electric current (below 100 μ A). However, under these conditions Tyr co-migrated with levodopa. Subsequently, the concentration of sulfated β -CD was increased which resulted in the separation of both compounds (Fig. 2). At a CD concentration of 8 mg/mL good resolution was achieved for all compounds. In addition, the enantiomers of 6-OH-DOPA were resolved using a CD concentration of 4 mg/mL or higher. Substituting sodium phosphate buffer with a phosphoric acid/triethanolamine buffer, pH 2.0 resulted in a noisy baseline and was therefore not further investigated. Interestingly, when the phosphate buffer was prepared from orthophosphoric acid and sodium hydroxide solution, buffer molarity and sulfated β -CD concentration could be reduced to 0.1 M and 6 mg/mL, respectively, with comparable resolution to the conditions shown in Fig. 2D. Increasing the applied voltage leads to currents above 100 μ A and, moreover, partial overlapping between Tyr and D-DOPA was observed. Therefore, the current was kept at 20 kV. Finally, the capillary was shortened to 35 cm effective length. Thus, the optimized conditions employed 0.1 M phosphate buffer, pH 2.0, prepared from orthophosphoric acid and sodium hydroxide solution containing 6 mg/mL of sulfated β -CD as background electrolyte using a 50 μ m id fused-silica capillary with an effective length of 35 cm (total length 45.2 cm) at an applied voltage of 20 kV and 18 °C capillary temperature. The separation of a sample containing 2 mg/mL levodopa and 0.5% of the impurities (also containing the internal standard Phe) is shown in Fig. 3.

Due to the fact that a randomly substituted sulfated β -CD as a commercially available and low priced CD derivative was selected in the present study two batches of this CD from another supplier (CD2 and CD3) were tested with

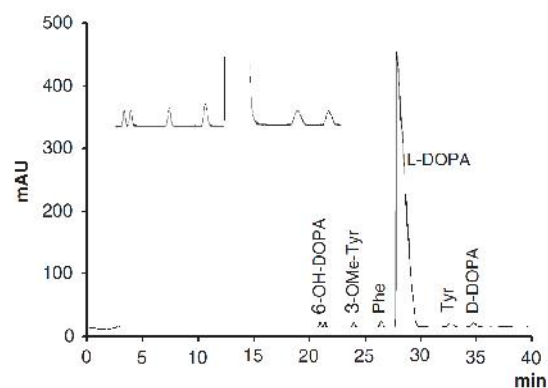
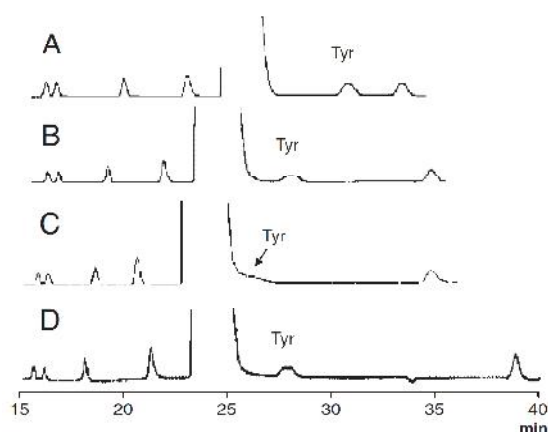


Figure 3. Electropherogram of 2 mg/mL levodopa containing 0.5% of the impurities and 20 μ g/mL of Phe as internal standard. Experimental conditions: 0.1 M phosphate buffer, pH 2.0 containing 6 mg/mL sulfated β -CD; 45.2/35 cm fused-silica capillary, 20 kV, 18 °C.

regard to the reproducibility of the separation of the analytes in addition to the CD used for method development (CD1) (Fig. 4). Clearly, the different samples of sulfated β -CD affected the separation, especially the critical resolution between levodopa and Tyr. CD1 and CD2 provided sufficient resolution for quantitation at a CD concentration of 6 mg/mL while Tyr migrated on the tailing of the peak of levodopa when using CD3. However, increasing the concentration of CD3 to 8 mg/mL resulted in a baseline resolution between levodopa and Tyr (Fig. 4D). Using a sample containing 10 μ g/mL of levodopa and Tyr at CD concentrations of 6 mg/mL a resolution $R_s = 5.8$ was determined for CD1, $R_s = 4.3$ for CD2 and $R_s = 1.8$ for CD3. As $R_s = 4.2$ resulted in baseline separation at a concentration of 2 mg/mL levodopa containing 0.5% Tyr, a

Table 1. Calibration data of impurities at a concentration of 2 mg/mL levodopa

Compound		Slope	Intercept	Correlation coefficient (r^2)	Residual sum of squares
6-OH-DOPA	Enantiomer 1	0.8094	−0.0011	0.9902	0.0059
	Enantiomer 2	0.8368	−0.0129	0.9918	0.0050
3-OMe-Tyr		0.6083	0.0054	0.9932	0.0041
Tyr		0.7425	0.0199	0.9956	0.0026
D-DOPA		0.5402	0.0435	0.9978	0.0014

**Figure 4.** Effect of different batches of sulfated β -CD on the separation of the analytes. Experimental conditions: 0.1 M phosphate buffer, pH 2.0, containing (A) 6 mg/mL CD1, (B) 6 mg/mL CD2, (C) 6 mg/mL CD3, and (D) 8 mg/mL CD3. Other conditions and sample concentration as in Fig. 3.

minimum resolution of $R_s = 4.0$ between levodopa and Tyr appears to be necessary for the quantitation of all impurities. This should be evaluated as a system suitability test before application of the CF method.

3.2 Method validation

The optimized method was validated according to the ICH guideline Q2 (R1) [24] (<http://www.ich.org>) with regard to specificity, linearity, range, intra-day precision, inter-day precision, LOD, and LOQ for the impurities in the presence of 2 mg/mL levodopa. As D-DOPA was not available, the samples were spiked with racemic DOPA. Phe was selected as internal standard with a closely related structure in order to compensate for injection errors and fluctuations of the migration time. The concentration of Phe was 20 μ g/mL. No interfering system peaks were observed in blank samples. Linearity was determined in the range of 0.1 (2 μ g/mL)–1.0% (20 μ g/mL). The data from the calibration graphs are summarized in Table 1. Correlation coefficients were at least 0.99 indicating sufficient linearity in the investigated concentration range. The 95% confidence intervals of the y -intercept included zero for all compounds. Thus, a

Table 2. LOD and LOQ of specified impurities of levodopa expressed as percent of levodopa based on the concentration of 2 mg/mL

Compound		LOD		LOQ	
		μ g/mL	%	μ g/mL	%
6-OH-DOPA	Enantiomer 1	0.8	0.04	2.0	0.1
	Enantiomer 2	0.8	0.04	2.0	0.1
3-OMe-Tyr		0.6	0.03	2.0	0.1
Tyr		0.6	0.03	2.0	0.1
D-DOPA		0.8	0.04	2.0	0.1

Table 3. Intraday and interday precision expressed as RSD values (intraday $n = 6$, interday $n = 3$)

Compound		Level (%)	Intraday	Interday
6-OH-DOPA	Enantiomer 1	0.1	9.05	10.09
		0.9	3.75	4.55
	Enantiomer 2	0.1	3.99	9.34
		0.9	3.73	4.88
3-OMe-Tyr		0.1	7.94	7.97
		0.9	1.73	9.31
Tyr		0.1	6.11	7.67
		0.9	1.95	5.25
D-DOPA		0.1	4.08	6.36
		0.9	2.09	6.11

systematic error can be excluded. The LOD and LOQ of the impurities in the presence of 2 mg/mL levodopa as summarized in Table 2 corresponded to a signal-to-noise ratio of 3 and 10, respectively. Precision was determined by injecting concentrations at the 0.1 and 0.9% level six times on one day (intraday precision) and three times on three consecutive days (interday precision). The results summarized in Table 3 demonstrate sufficient precision of the method. At the LOQ of 0.1% RSD values of 10% or better were observed.

3.3 Analysis of drug substance

The method was applied to determine the related substances as well as D-DOPA in samples of levodopa including the

Table 4. Determination of impurities in samples of levodopa; the amount is expressed as percent

Compound		Sample 1	Sample 2	Sample 3
6-OH-DOPA	Enantiomer 1	ND ^{a)}	ND	ND
	Enantiomer 2	ND	ND	ND
3-OMe-Tyr		ND	ND	ND
Tyr		0.07 ^{b)}	0.05 ^{b)}	0.09 ^{b)}
D-DOPA		0.08 ^{b)}	0.15 ± 0.01	ND

a) Not detectable.

b) Estimated value because below LOQ.

chemical reference substance of the European Pharmacopoeia (sample 2). All samples were investigated at a concentration of 2 mg/mL, the concentration of the internal standard Phie was 20 µg/mL. The results are summarized in Table 4. Neither 6-OH-DOPA nor 3-OMe-Tyr could be detected in the samples although sample 2 showed a very small peak with the migration time of 3-OMe-Tyr but as the signal-to-noise ratio of this peak was below 3 the amount is, therefore, below the LOD this was not considered. Tyr was detected in all samples but the quantities were below the LOQ and could therefore only be estimated. No other peaks were observed in the samples. Calculating the amount of D-DOPA in sample 2 as percentage of the total of the corrected peak areas of all peaks (normalization procedure of the European Pharmacopoeia) a value of $0.12 \pm 0.01\%$ was obtained. This is in reasonable agreement with the value determined using the internal standard as reference.

The European Pharmacopoeia limits the related substances by HPLC to 0.1% for 6-OH-DOPA, to 0.2% for 3-OMe-Tyr, and to 0.5% for Tyr as well as D-DOPA in an enantioselective HPLC assay to 0.5%. The limits of the United States Pharmacopoeia are 0.5% for 3-OMe-Tyr and 0.1% for all other related substances. D-DOPA is determined by optical rotation, which is not considered here. From the current CE data it can be concluded that the investigated samples all comply with the pharmacopoeial requirements.

4 Concluding remarks

A CE method has been developed and validated for the determination of the impurities including the (R)-enantiomer D-DOPA. The method allowed the quantitation of the impurities at the 0.1% level. Compared to reports on the analysis of the enantiomeric purity of levodopa by CE in the literature lower LOD and LOQ values were reported by most studies in the range of 0.015–0.05% for LOD and 0.04–0.17% for LOQ [12–14, 16]. This may be attributed to the relatively low sensitivity of the diode-array detector of the CE instrument used in the present study. Nevertheless, the present method appears to be suitable for the intended

purpose of the simultaneous analysis of all impurities in levodopa.

As demonstrated by many other studies, the use of randomly substituted CDs bears the problem of insufficient reproducibility of separations when switching from one supplier to another or between different batches from one supplier. However, this may be compensated by introduction of a system suitability test assuring sufficient resolution between a critical pair of analytes by adjusting the concentration of the chiral selector. General suggestions for the range of the factors affecting a CE separation such as composition of the background electrolyte, buffer additives, capillary length, applied voltage, etc. that can be adjusted to achieve sufficient resolution for a critical pair have been published by Holzgrabe et al. [13].

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The authors have declared no conflict of interest.

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Manuscript V

Development and validation of a capillary electrophoresis assay for the determination of stereoisomeric purity of chloroquine enantiomers

Sudaporn Wongwan, Gerhard K.E. Scriba, *submitted manuscript*.

Development and validation of a capillary electrophoresis assay for the determination of the stereoisomeric purity of chloroquine enantiomers

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Abbreviations: **SBE- β -CD**, sulfobutylether- β -cyclodextrin;

Abstract

A stereoselective CE assay for the determination of the enantiomeric purity determination of *R*-(-)-chloroquine and *S*-(+)-chloroquine was developed and validated. The separations were performed in a 50/40.2 cm uncoated fused-silica capillary at 20°C using a 100 mM sodium phosphate buffer, pH 2.5, containing 30 mg/mL sulfobutylether(VII)- β -cyclodextrin as background electrolyte operated at an applied voltage of -25 kV and 20°C. The detection wavelength was 225 nm. Carbamazepine was used as internal standard. The assay was validated in the range of 0.05 - 1.0 % for the respective minor chloroquine enantiomer based on a concentration of 3 mg/mL of the major enantiomer, either *R*-(-)-chloroquine or *S*-(+)-chloroquine. The method was applied to analyze the stereoisomeric purity of synthetic samples of the chloroquine enantiomers.

Keywords: Chloroquine, Capillary electrophoresis, Cyclodextrin, Enantiomeric purity, Impurity profiling

1. Introduction

Chloroquine (7-chloro-4-(4-diethylamino-1-methylbutylamino)quinoline, Figure 1) is a widely used chemotherapeutic drug for the prophylaxis and the treatment of malaria [1-3]. The drug is active only against the intra-erythrocytic forms of the life cycle of the *Plasmodium* parasite. Although the molecular mechanism of the antischizontal activity is not yet completely understood it appears to be associated with the interaction between heme and chloroquine [1, 2, 4]. Heme polymerization in the food vacuole of the parasite is inhibited by complexation of chloroquine by heme leading to the accumulation of toxic heme. Furthermore, there is evidence that the formation of the heme-chloroquine complex inhibits the catalase activity of heme resulting in accumulation of H₂O₂ and an increased oxidative stress which can lead to damage of lipids and proteins. Chloroquine has also been used as an anti-inflammatory drug for the treatment of rheumatoid arthritis [5, 6].

Chloroquine is commercially available as racemate. While there is no significant difference of the activity of the individual enantiomers against *Plasmodium falciparum* in vitro [7], differences in the antimalarial activity and toxicity of the enantiomers have been reported in vivo [8, 9]. This may be due to stereoselective metabolism of the drug. Furthermore, the enantiomers appear to be less embryotoxic compared to the racemate [10]. Due to these controversial data it appears desirable to further study the enantioselective effects of the drug. Such efforts unequivocally require suitable assays for the determination of the purity of the enantiomers of the compound.

The analytical separation of the chloroquine enantiomers has been achieved by HPLC using a chiral stationary phase containing α 1acid glycoprotein [11, 12] or heparin as chiral selector [13]. Capillary electrophoresis (CE) has become a powerful analytical microseparation technique in recent years that is especially suited for enantioseparations [14-19]. The CE enantioseparation of chloroquine has been achieved in the presence of various CDs as chiral selectors including sulfated β -CD [20-22], sulfobutylether- β -CD (SBE- β -CD) [23] or a mixture of γ -CDs [24]. Furthermore chloroquine enantioseparations in the presence of the polysaccharide colominic acid [25], human serum albumin [26] and arsenyl-L-(+)-tartrate [27] have been published. However, none of the CE assays has been used for the determination of the stereoisomeric purity of the chloroquine enantiomers. Therefore, the aim of the present study was to develop and validate the stereoselective CE assay for the determination of synthetic chloroquine enantiomers.

2. Materials and methods

2.1 Chemicals

All chemicals were of analytical grade. Chloroquine diphosphate, carbamazepine, sulfated β -cyclodextrin were from Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany). Sulfobutylether(V)- β -cyclodextrin (SBE(V)- β -CD) and sulfobutylether(VII)- β -cyclodextrin (SBE(VII)- β -CD) with the degrees of substitution ca. 4.9 and 6.6, respectively, were obtained from CyDex Pharmaceuticals, Inc. (Lexexa, KS, USA). Sodium hydroxide solution was from Fisher Scientific (Schwerte, Germany) and phosphoric acid was from Carl Roth GmbH (Karlsruhe, Germany). (*R*)-(-)-chloroquine phosphate was synthesized according to [28]. All buffers and solutions were prepared in deionized, double-distilled water, filtered through 0.2 μ m-filter, and degassed by sonication before used.

2.2 CE

The separations were performed using a Beckman Coulter MDQ Instrument (Fullerton, CA, USA) equipped with an UV detector set at 225 nm. A 40/50.2 cm, 50 μ m id fused-silica capillary thermostated at 20°C was used. A new capillary was treated with 1.0 M sodium hydroxide for 20 min, 0.1 M hydrochloric acid for 10 min, water for 10 min and sodium phosphate buffer for 10 min. Between the injections, the capillary was flushed with 0.1 M sodium hydroxide for 3 min, 0.1 M phosphoric acid for 2 min, water for 2 min, and the sodium phosphate buffer for 8 min. Samples were introduced by hydrodynamic injection at 0.5 psi for 6 s. The applied voltage was –25 kV (reversed polarity, detection at the anode).

The optimized background electrolyte consisted of a 100 mM sodium phosphate buffer prepared by adjusting 100 mM phosphoric acid to pH 2.5 with 1 M sodium hydroxide solution. CDs were dissolved in the buffer solution after the adjustment of the pH. The background electrolyte was replaced after three analyses. Stock solutions of 1.0 mg/mL of racemic chloroquine phosphate and (*R*)-(–)-chloroquine were prepared in water. The stock solution of 1.0 mg/mL of the internal standard carbamazepine was prepared in methanol. The solutions were subsequently diluted with water to the desired concentrations.

2.3 Method validation

Method validation was conducted according to the ICH guideline Q2(R1) [29] with regard to range, linearity, limit of detection and quantitation, and precision. Linearity was estimated in the range of 3.0 μ g/mL to 60 μ g/mL of racemic chloroquine corresponding to 1.5 μ g/mL to 30 μ g/mL for the individual enantiomers by unweighted linear regression using the least-square method. The limit of detection and limit of quantitation were based on signal to noise ratio 3:1 and 10:1, respectively. Precision was determined at a low concentration (3.0 μ g/mL) and a high concentration (24 μ g/mL) of the enantiomers. Intraday precision was calculated from six replicate injections in the same day while interday precision was based on six injections on three consecutive days.

3. Results and discussion

3.1 Method development

The separation of the chloroquine enantiomers (Figure 1) has been reported using charged CD derivatives as chiral selectors in acidic background electrolytes [20-23] as well as less frequently used additives such as human serum albumin [26], colominic acid [25] or arsenyl-*L*-(+)-tartrate [27]. Due to the commercial availability sulfated β -CD and SBE- β -CD were evaluated. Initial experiments were conducted using sulfated β -CD in concentrations between 5 and 30 mg/mL in a 50 mM sodium phosphate buffer, pH 2.5. At concentrations of sulfated β -CD as low as 5 mg/mL the compounds could not be detected applying a voltage of 15 - 20 kV under normal polarity. In contrast, the compounds were detected upon reversing the polarity of the applied voltage. However, significant peak broadening was observed under these conditions as shown in Figure 2A. Increasing the concentration of the background electrolyte to 100 mM significantly improved the peak shape (Figure 2B). Under these conditions the generated current was acceptable (< 100 μ A). However, upon injection of a concentrated sample containing 2 mg/mL (*R*)-(–)-chloroquine and

about 20 $\mu\text{g/mL}$ (S)-(+)-chloroquine resulted in a severely distorted peak of the minor enantiomer which could not be improved increasing the concentration of sulfated β -CD or the background electrolyte or variation of the applied voltage (results not shown).

Subsequently, two batches of SBE- β -CD varying in the degree of substitution were evaluated. While no enantioseparation was found in case of the CD with a degree of substitution of 5 (SBE(V)- β -CD), resolution of the chloroquine enantiomers was observed using the CD derivative with an average substitution of 6.6 (SBE(VII)- β -CD) under reversed polarity of the applied voltage (Figure 2C). It is well known that the degree of substitution of SBE- β -CD can affect stereoisomer separations in CE [30-32]. Compared to sulfated β -CD, reversal of the enantiomer migration order was noted. Increasing the concentration of SBE(VII)- β -CD to 30 mg/mL allowed to raise the concentration of the chloroquine sample to 3 mg/mL which resulted in an improved LOD of the enantiomeric impurity. Thus, the optimized conditions employed a 100 mM sodium phosphate buffer, pH 2.5, containing 30 mg/mL SBE(VII)- β -CD as the background electrolyte a 50 μm id fused-silica capillary with an effective length of 40 cm at an applied voltage of -25 kV and 20°C . Under these conditions the current ranged between 80 and 90 μA . The total run time was 20 min. The analytes were detected at 225 nm. Several compounds were evaluated as internal standard including structurally related compounds such as quinine. However, the compound was not detected within 30 min under reversed polarity conditions. Finally, carbamazepine was selected as internal standard which has a migration time of about 6 min.

3.2 Method validation

The optimized method was validated according to the ICH guideline Q2(R1) [29] regarding linearity, range, LOD, LOQ as well as intraday and interday precision. As pure enantiomers of chloroquine were not available, racemic drug was used for method validation. A range of 1.5 - 30 $\mu\text{g/mL}$ of the enantiomers was selected which corresponds to a relative concentration of the minor enantiomer of 0.05 % to 1.0 % based on a concentration of the major enantiomer of 3 mg/mL. Carbamazepine at a concentration of 40 $\mu\text{g/mL}$ was used as internal standard to compensate for minor injection errors and fluctuation of the migration times. The calibration data obtained by unweighted linear regression are summarized in Table 1. Correlation coefficients of at least 0.997 were observed. The 95 % confidence intervals of the y -intercepts included zero so that a systematic error can be excluded. The LOQ was 1.5 $\mu\text{g/mL}$ (0.05 %) for both enantiomers and the LOD was 0.6 $\mu\text{g/mL}$ (0.02 %). Intraday and interday precision determined at the 0.1 % (3 $\mu\text{g/mL}$) and 0.8 % level (24 $\mu\text{g/mL}$) was generally below 6 %.

3.3 Sample analysis

The method was applied to the determination of the enantiomeric purity of a synthetic sample of (R)-(-)-chloroquine prepared according to a published method [28]. The electropherogram of a sample containing 3.0 mg/mL (R)-(-)-chloroquine and 40 $\mu\text{g/mL}$ carbamazepine as internal standard is shown in Figure 3. $0.24 \pm 0.01\%$ (mean \pm SD, $n = 3$) of the (S)-enantiomer could be detected corresponding to an enantiomeric purity of 99.52 % of the synthetic sample. Using peak

normalization the amount of the (*S*)-enantiomer was 0.35 ± 0.01 % (mean \pm SD, $n = 3$). In addition to (*S*)-(+)-chloroquine another impurity (peak X in Figure 3) migrating at about 13 min could be observed in the laboratory sample. The synthetic precursor 4,7-dichloroquioline has a migration time of 7.5 min (data not shown) so that the structure of peak X cannot be assigned currently. Using peak normalization the impurity amounts to about 0.41 ± 0.02 %.

4. Concluding remarks

A stereoselective CE assay was developed and validated for the determination of enantiomeric purity of synthetic (*R*)-(-)-chloroquine and (*S*)-(+)-chloroquine using SBE(VII)- β -CD as chiral selector. Interestingly, enantioresolution could not be achieved in the presence of a lower substituted SBE- β -CD derivative further illustrating the effect of the substitution of SBE- β -CD on chiral separations [30-32]. The optimized method allowed the quantitation of the stereoisomeric impurity at the 0.05% level. In a laboratory sample of (*R*)-(-)-chloroquine about 0.24 % of the enantiomeric impurity was observed. In addition, an unknown impurity at the 0.4 % level could be detected. Thus, the present CE method may also be suitable for impurity profiling of chloroquine.

Acknowledgements

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5. References

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Table 1. Calibration data including intraday and interday precision expressed as RSD values (intraday n = 6; interday n = 3)

Parameters	(R)-(-)-Chloroquine	(S)-(+)-Chloroquine
Range [$\mu\text{g/mL}$]	1.5 – 30	1.5 – 30
[% ¹⁾]	0.05 – 1.0	0.05 – 1.0
Slope	2.0409	2.2044
Intercept	-0.0287	-0.0409
Correlation coefficient r	0.9985	0.9974
Residual sum of squares	0.0022	0.0038
95% confidence interval of intercept	-0.0744 – 0.0170	-0.1023 – 0.0205
Intraday precision (%RSD)		
Low level (0.3 $\mu\text{g/mL}$ / 0.1%)	3.57	3.30
High level (24 $\mu\text{g/mL}$ / 0.8%)	4.75	3.34
Interday precision (%RSD)		
Low level (0.3 $\mu\text{g/mL}$ / 0.1%)	5.78	3.37
High level (24 $\mu\text{g/mL}$ / 0.8%)	4.83	4.26

¹⁾ Percent corresponding to a concentration of 3 mg/ml (-)-chloroquine or (+)-chloroquine.

Figure legends

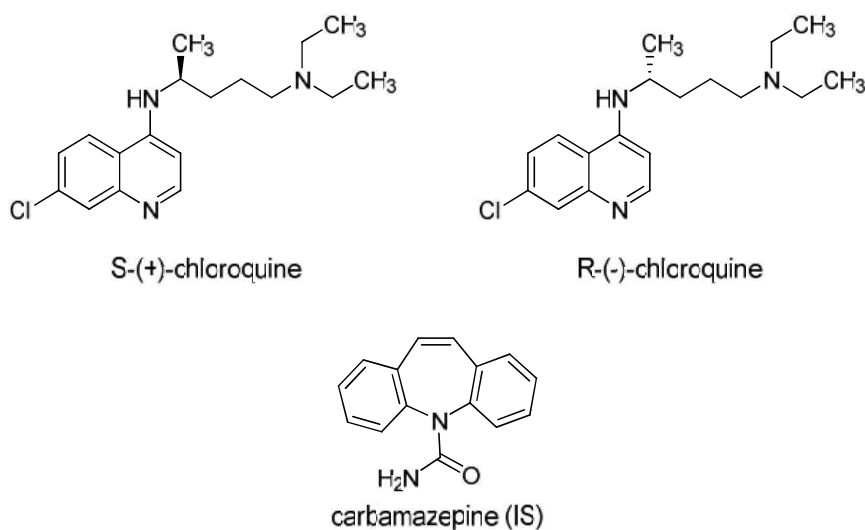


Figure 1. Structures of (*R*)-(-)-chloroquine and (*S*)-(+)-chloroquine as well as carbamazepine (internal standard).

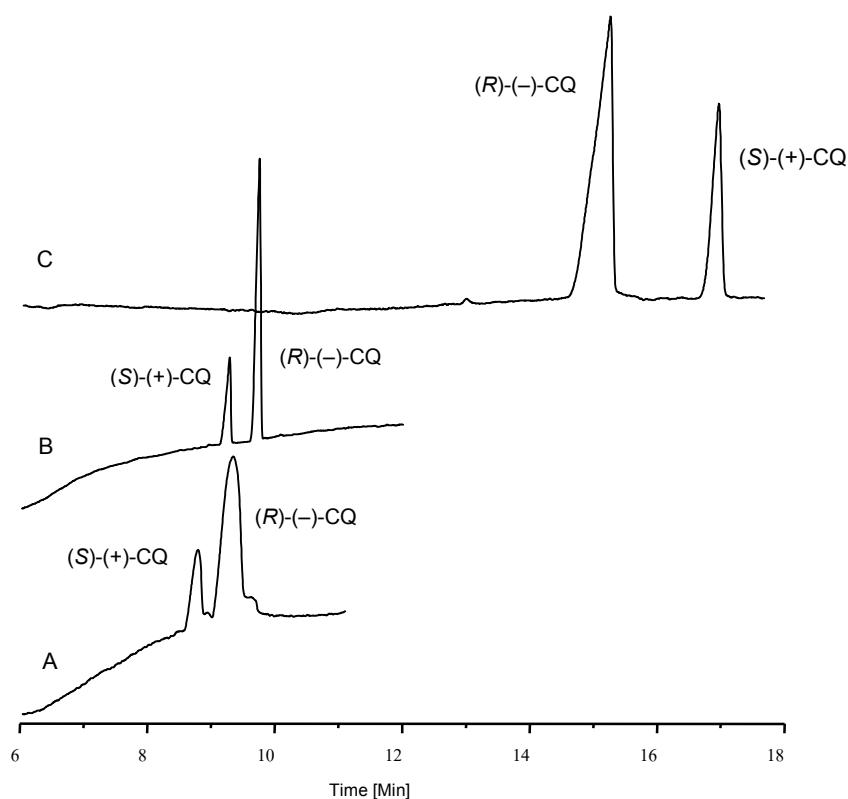


Figure 2. Electropherograms of the separation of a non-racemic mixture of chloroquine (400 $\mu\text{g/mL}$ (*R*)-(-)-chloroquine ((*R*)-(-)-CQ) and 200 $\mu\text{g/mL}$ (*S*)-(+)-chloroquine ((*S*)-(+)-CQ)) using sulfated β -CD (A and B) and SBE(VII)- β -CD (C) as chiral selector. Experimental conditions: 50.2/40 cm, 50 μm id fused-silica capillary, 20°C, 225 nm, (A) 50 mM sodium phosphate buffer, pH 2.5, 20 mg/mL sulfated β -CD, -15 kV, (B) 100 mM sodium phosphate buffer, pH 2.5, 20 mg/mL sulfated β -CD, -15 kV and (C) 100 mM sodium phosphate buffer, pH 2.5, 20 mg/mL SBE(VII)- β -CD, -25 kV.

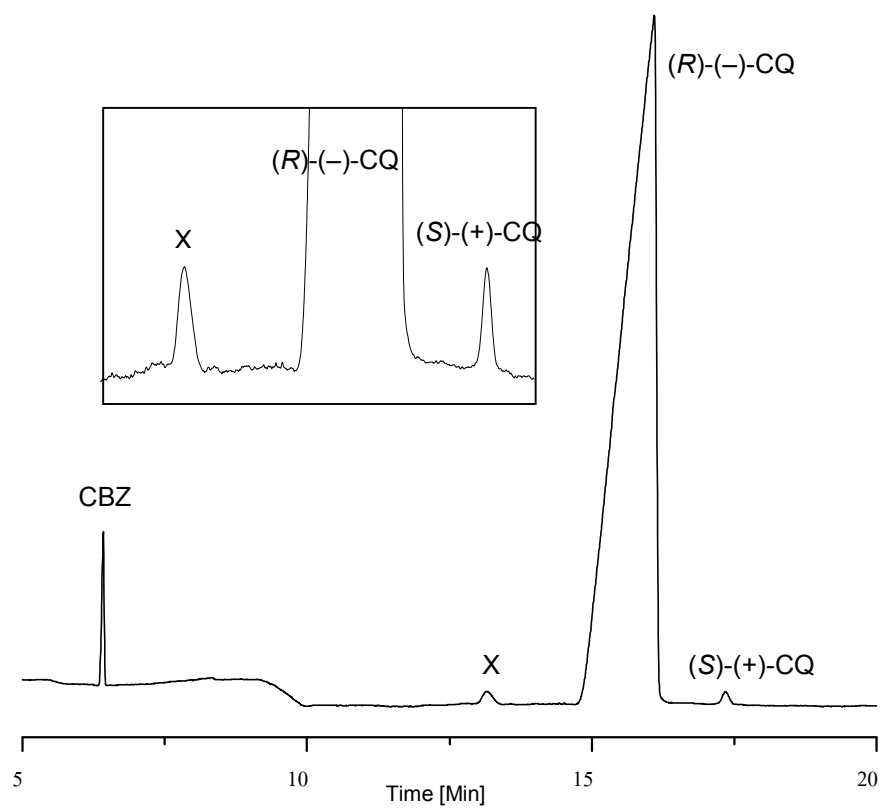


Figure 3. Electropherogram of a sample containing 3 mg/mL (*R*)-(-)-chloroquine ((*R*)-(-)-CQ) with about 0.3% (*S*)-(+)-chloroquine ((*S*)-(+)-CQ) as well as 40 μ g/mL carbamazepine (CBZ, IS) using the optimized method. Experimental conditions: 50.2/40 cm, 50 μ m id fused-silica capillary, 100 mM sodium phosphate buffer, pH 2.5, 30 mg/mL SBE(VII)- β -CD, -25 kV, 20°C, 225 nm.

CHAPTER III

DISCUSSION

The discussion is divided into four parts. (1) In this chapter the stereoselective CE assays for simultaneous determination of charged and neutral impurities in dexamphetamine sulfate are discussed. A comparison of two cyclodextrin-mediated capillary zone electrophoresis (CD-mediated CZE) assays as well as cyclodextrin-modified microemulsion electrokinetic chromatography (CD-modified MEEKC) assay in both efficiency of the methods and cost analysis is described. (2) A stereoselective CE assay for the impurity profiling of levodopa is discussed. (3) A discussion regarding the determination of enantiomeric purity of chloroquine enantiomers as another example for the applications of stereoselective CE assay is included. (4) Subsequently, the method development and optimization of all present CE assays are discussed together. A schematic flow chart showing the strategies for accomplishing stereoisomeric separations of compounds containing basic and neutral functional groups is developed.

3.1 Stereoselective CE assays for the impurity profiling of dexamphetamine sulfate drug substances (Manuscript I, II and III)

3.1.1 Profiling of levoamphetamine and related substances in dexamphetamine sulfate by capillary electrophoresis (Manuscript I)

Dexamphetamine (dextroamphetamin, 2S-(+)-amphetamine, (S)-1-phenyl-2-propanamine, Fig. 1) and amphetamine are controlled substances in most European countries and the United States of America. Dexamphetamine is a central stimulant therapeutically used in the treatment of the attention deficit hyperactivity disorder of children [56, 57] and narcolepsy [58]. Dexamphetamine sulfate is described in the monographs of the United State Pharmacopeia 32 (USP 32) [53] and the British Pharmacopoeia 2009 (BP 2009) [54]. The enantiomeric purity of dexamphetamine sulfate is characterized by the optical rotation in both pharmacopeias. Besides, the USP also determines the chromatographic purity limiting individual impurities to 0.1% and the total impurities to 0.5%. No impurities are specified. As the sensitivity of a simple polarimetric measurement is low, a stereoselective CE assay was developed in order to obtain the sensitive method as well as the simultaneous determination of related substances and enantiomeric impurity in dexamphetamine sulfate. Dexamphetamine can be prepared from the natural compounds such as 1R,2S-(–)-norephedrine or 1S,2S-(+)-norpseudoephedrine [59, 60]. Thus, levoamphetamine as well as the chiral β -hydroxy-phenylethylamines was included as potential charged impurities in dexamphetamine sulfate. The structures of dexamphetamine and potential impurities are shown in Fig. 1.

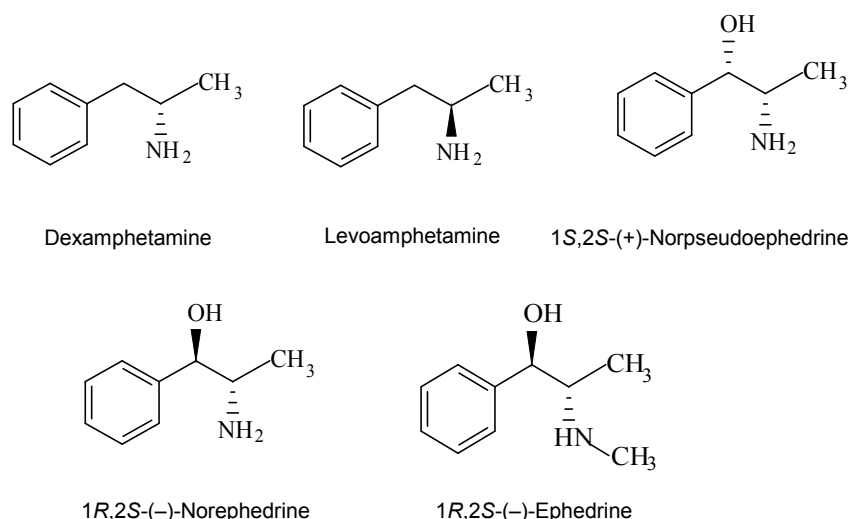


Figure 1. Structures of dexamphetamine, levoamphetamine, 1R,2S-(-)-norephedrine, 1S,2S-(+)-norpseudoephedrine and the internal standard 1R,2S-(-)-ephedrine.

The CE assay for the enantioseparation of amphetamine has been reported in several studies employing native cyclodextrins as well as neutral and charged CD derivatives as chiral selectors [61–64]. Consequently, initial screening tests were performed using a 50 mM phosphate buffer phosphate buffer, pH 2.5, containing β -CD, 2-hydroxypropyl- β -CD, 2,6-dimethyl- β -CD, 2,3,6-trimethyl- β -CD, carboxymethyl- β -CD, succinyl- β -CD, sulfated- β -CD and heptakis-(2,3-di-O-acetyl-6-O-sulfo)- β -CD (HDAS- β -CD) as the background electrolyte. Good resolution ($R_s > 2$) was obtained with sulfated- β -CD and HDAS- β -CD at concentrations of 1–3 mg/mL. However, only HDAS- β -CD was selected for method optimization due to better peak shape and the fact that HDAS- β -CD is a single isomer derivative. Therefore, the batch-to-batch reproducibility will not affect the performance of the assay. Subsequently, the buffer pH was increased from 2.5 to 3.5 and 6.5 which led to shorter migration time but also poorer peak shape. Thus, the buffer pH at 2.5 was considered to be the optimum. Upon injection of a sample containing dexamphetamine sulfate at a concentration of 2 mg/mL, peak deformation of the later migrating levoamphetamine was detected. Increasing the CD concentration from 2 mg/mL to 10 mg/mL as well as the buffer molarity from 50 mM to 100 mM led to acceptable peak shapes of all analytes with an analysis time of less than 15 min (Fig. 2A). Peak deformation of the minor peak of levoamphetamine may be caused by the extensive CD “consumption” by the predominant peak of dexamphetamine. Thus, increasing the CD concentration can improve the peak shape of levoamphetamine. It was observed that a further increase in CD concentration up to 20 mg/mL led to a loss of selectivity. Therefore, the optimized background electrolyte consisted of a 0.1 M phosphate buffer, pH 2.5, containing 10 mg/mL of HDAS- β -CD. The separation was carried out in a 50 μ m id fused-silica capillary with an effective length of 46.5 cm at an applied voltage of 25 kV with electric currents of about 90 μ A and operated at capillary temperature 20°C. All compounds were detected at 205 nm. The electropherogram of a standard solution of 2 μ g/mL of 1R,2S-(-)-norephedrine and 1S,2S-(+)-norpseudoephedrine as well as 1.2 μ g/mL of dexamphetamine and levoamphetamine containing 75 μ g/mL of ephedrine (as internal standard) is shown in Fig. 2B.

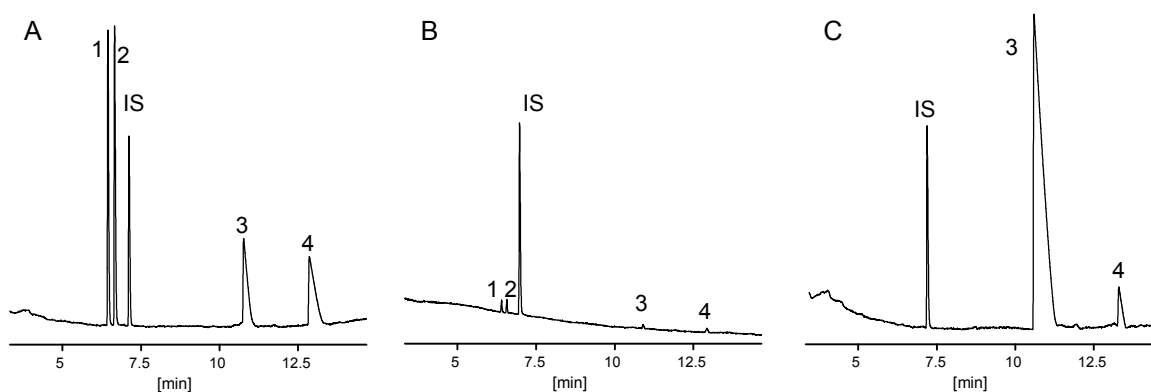


Figure 2. Electropherograms of (A) separation of standards (75-144 $\mu\text{g/mL}$), (B) standards at 2 $\mu\text{g/mL}$ (1*R*,2*S*-(-)-norephedrine and 1*S*,2*S*-(+)-norpseudoephedrine) and 1.2 $\mu\text{g/mL}$ (dexamphetamine and levoamphetamine) containing 75 $\mu\text{g/mL}$ of ephedrine and (C) commercial sample of 2.0 mg/mL dexamphetamine sulfate containing 3.8% levoamphetamine. (1) 1*S*,2*S*-(+)-norpseudoephedrine, (2) 1*R*,2*S*-(-)-norephedrine, (IS) 1*R*,2*S*-(-)-ephedrine, (3) dexamphetamine, (4) levoamphetamine. Experimental conditions: 0.1 M sodium phosphate buffer, pH 2.5, containing 10 mg/mL HDAS- β -CD; 51/46.5 cm, 50 μm id fused-silica capillary; 25 kV; 20°C.

The optimized method was subsequently validated according to the ICH guideline “Validation of analytical procedures”, Q2(R1) [52], with regard to linearity, range, limit of detection (LOD), limit of quantitation (LOQ) as well as intraday and interday precision. Method validation was performed using the racemic mixture of amphetamine because enantiomerically pure dexamphetamine was not available. Calibration data performed over a range of 1.2-100 $\mu\text{g/mL}$ corresponding to 0.06-5% based on a concentration of 2 mg/mL dexamphetamine sulfate. Correlation coefficients of at least 0.995 were observed indicating sufficient linearity in the investigated concentration range. The 95% confidence intervals of the y-intercepts included zero for all analytes, so that a systematic error could be excluded. The LOQ estimated at a signal-to-noise ratio of 10 was 0.06% (1.2 $\mu\text{g/mL}$) for all impurities. The LOD estimated at a signal-to-noise ratio of 3 was 0.02% (0.4 $\mu\text{g/mL}$) for 1*R*,2*S*-(-)-norephedrine and 1*S*,2*S*-(+)-norpseudoephedrine and 0.03% (0.6 $\mu\text{g/mL}$) for levoamphetamine. The repeatability of the method was expressed by the relative standard deviation (RSD) values of the peak area ratios between the analyte and the internal standard. The RSD values of intraday and interday precision at high and low concentrations were below 7% for all impurities indicating a sufficient repeatability of the method.

Samples containing 2 mg/mL of commercial dexamphetamine sulfate drug substance and 75 $\mu\text{g/mL}$ of the internal standard were subsequently analyzed employing the validated method. Neither 1*R*,2*S*-(-)-norephedrine nor 1*S*,2*S*-(+)-norpseudoephedrine could be detected in the samples. In contrast, the content of levoamphetamine ranged between 2.8 and 4.0% (Fig. 2C, Table 1). Comparable concentrations of levoamphetamine were determined by peak area normalization as shown in Table 1. The data indicated that the investigated dexamphetamine sulfate batches were not synthesized starting from the natural compounds, 1*R*,2*S*-(-)-norephedrine or 1*S*,2*S*-(+)-norpseudoephedrine, but may be obtained by other synthetic routes which lead to racemic amphetamine such as reductive amination starting from 1-phenyl-2-propanone. The racemic amphetamine is subsequently resolved by fractionated crystallization.

Table 1. Content of levoamphetamine in commercial samples of dexamphetamine sulfate drugs substances (means \pm SD, n = 3)

Sample	[%] peak area ratios	[%] peak area normalization
1	3.79 ± 0.12	4.13 ± 0.22
2	2.79 ± 0.23	2.89 ± 0.13
3	3.97 ± 0.15	4.03 ± 0.05
4	2.88 ± 0.16	3.04 ± 0.35
5	3.26 ± 0.24	2.98 ± 0.26

A LOQ of 0.5% was reported for a chiral HPLC assay employing chiral stationary phase [65]. Thus, the present CE assay with a LOQ of levoamphetamine determination at the 0.06% level was rather sensitive. Moreover, levoamphetamine as well as related substances can be simultaneously analyzed. The determination of the enantiomeric purity of dexamphetamine sulfate sample 1 was also investigated by polarimetry according to the pharmacopeial tests of the USP 32 [53] and the BP 2009 [54]. A specific rotation of $[\alpha]_{\text{D}}^{20} = 21.5 \pm 0.43^\circ$ (n=3) was detected. The addition of 2% and 4% of racemic amphetamine into sample 1 resulted in values of $21.3 \pm 0.13^\circ$ and $21.1 \pm 0.17^\circ$, respectively. Therefore, it can be estimated that the dexamphetamine sulfate containing the *R*-enantiomer as high as 10% would still pass the tests of specific rotation as described in the monographs of the USP 32 ($[\alpha]_{\text{D}}^{25} = 20 - 23.5^\circ$) or the BP 2009 ($[\alpha]_{\text{D}}^{20} = 19.5 - 22^\circ$). Thus, the CE assay proved to be superior than using the test of specific rotation, since a dexamphetamine sample with an enantiomeric excess of as low as 80% would still meet the criteria of the test of the monographs.

3.1.2 CE assay for simultaneous determination of charged and neutral impurities in dexamphetamine sulfate using a dual CD system (Manuscript II)

Since none of the charged impurities (1*R*,2*S*-(-)-norephedrine and 1*S*,2*S*-(+)-norpseudoephedrine) could be detected in samples of commercial dexamphetamine sulfate drug substances, it was hypothesized that dexamphetamine was synthesized starting from phenylacetone (1-phenyl-2-propanone, benzyl methyl ketone) via Leuckart reaction or by reduction of phenylacetone oxime [66, 67] followed by fractional crystallization with *L*-(+)-tartaric acid [68]. Thus, charged as well as neutral starting materials or by-products as well as the enantiomer levoamphetamine may be present as related substances in dexamphetamine sulfate. Therefore, the aim of the study was the development and validation of a stereoselective CE assay for the determination of dexamphetamine allowing the simultaneous analysis of charged as well as neutral impurities. The structures of investigated compounds are shown in Fig. 3.

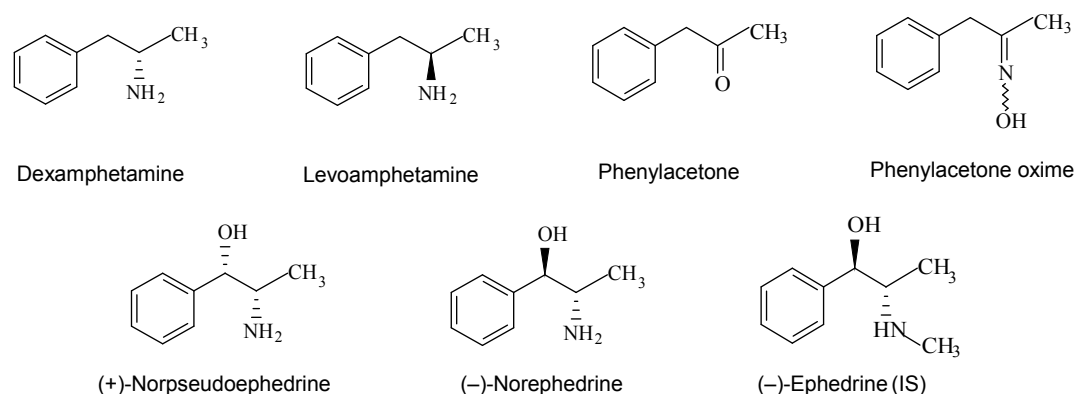


Figure 3. Structures of dexamphetamine, charged and neutral potential impurities as well as an internal standard, 1*R*,2*S*-(-)-ephedrine.

The enantioseparations of phenylalkylamines such as amphetamine, methamphetamine and ephedrine have been studied employing negatively charged CD derivatives including randomly sulfated CDs [64, 69–71] and sulfobutylether-CDs [69, 72]. Thus, various negatively charged CDs, *e.g.*, sulfated α -CD, sulfated β -CD, sulfated γ -CD, heptakis-(2,3-di-O-acetyl-6-O-sulfo)- β -CD, carboxymethyl- β -CD, carboxymethyl- γ -CD, sulfopropyl- β -CD, sulfopropyl- γ -CD and SBE(V)- β -CD dissolved in 50 mM phosphate buffer, pH 2.5, were investigated for their ability to separate the enantiomers of amphetamine and the neutral impurities phenylacetone and phenylacetone oxime. None of neutral impurities could be detected by any initial screening tests employing the normal polarity of the applied voltage. Therefore, reversed polarity allowing higher concentrations of the CDs that were intended to be used as a chiral selector as well as a carrier for neutral impurities was considered for the method development. The baseline separation of amphetamine enantiomers, phenylacetone and phenylacetone oxime was observed at reversed polarity of the applied voltage and a BGE containing sulfated β -CD or SBE(V)- β -CD (DS 4.9). Sulfated β -CD displayed high resolution of amphetamine enantiomers at a concentration of 20 mg/mL ($R_s > 3$) but none of neutral analytes could be detected within 60 min. Increasing the concentration of sulfated β -CD from 20 mg/mL to 40 mg/mL the neutral analytes migrated at about 45 min but the peaks were rather broad. In contrast, a high concentration of 80 mg/mL of SBE(V)- β -CD was necessary for baseline resolution of the amphetamine enantiomers (Fig. 4) but phenylacetone and its oxime migrated before amphetamine. At concentrations of 70 mg/mL of SBE(V)- β -CD or below broad peaks and/or peak splitting was observed (see also Fig. 4). It was observed that an increase in the concentration of SBE(V)- β -CD from 20 mg/mL to 80 mg/mL resulted in baseline separations and acceptable peak shapes of the amphetamine enantiomers as well as neutral impurities and a shorter migration time. Subsequently, a dual CD system comprising sulfated β -CD for the effective resolution of amphetamine enantiomers as shown in Fig. 4 and SBE(V)- β -CD for the separation of phenylacetone and phenylacetone oxime was selected.

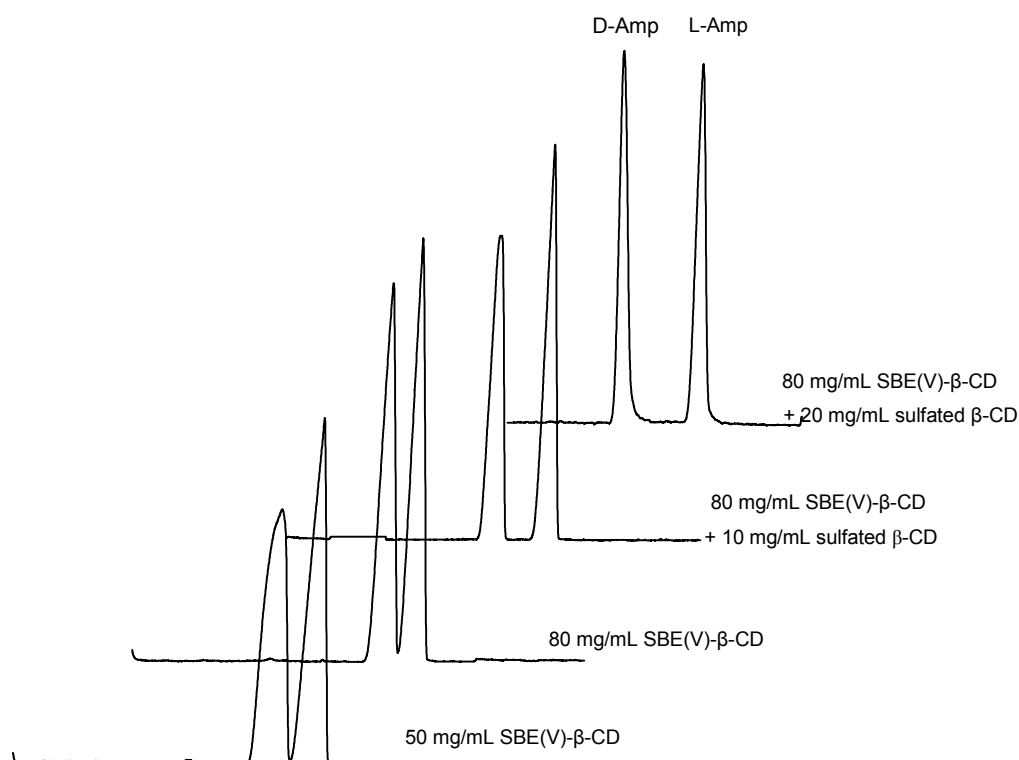


Figure 4. Effects of CD concentrations and compositions of the BGE to the separation of the amphetamine enantiomers (1 mg/mL) under otherwise identical conditions.

A concentration of 5 mg/mL of dexamphetamine sulfate was necessary in order to enable the determination of impurities at the 0.1% level. Thus, the concentration of sulfated β -CD in the presence of 80 mg/mL of SBE(V)- β -CD was varied between 10 and 30 mg/mL in order to achieve a baseline separation of all analytes. The background electrolyte composed of 25 mg/mL sulfated β -CD and 80 mg/mL of SBE(V)- β -CD resulted in an acceptable peak shape of all analytes (Fig. 5A). During the method development it was observed that phenylacetone oxime separated into two peaks when a concentration of SBE(V)- β -CD above 50 mg/mL was employed. Oximes can exist in two stereoisomers with *E* or *Z* configuration which can be distinguished, among others, by NMR spectroscopy. Subsequently, the phenylacetone oxime sample was analyzed by NMR spectroscopy and the present CE assay. The signals obtained from ^1H -NMR spectroscopy as well as the peak area ratio of 1:2.9 of the two peaks obtained from the CE assay were comparable with literature data. Thus, the two peaks can be assigned as phenylacetone *Z*-oxime and phenylacetone *E*-oxime, respectively (Fig. 5A). The partial overlap of the oximes could be overcome by increasing the buffer pH from 2.5 to 3.0 (Fig. 5B). It was observed that an increase in the buffer pH led to a long migration time as well.

Furthermore, the effective length of the capillary was shortened from 45 to 35 cm in order to reduce the analysis time. The applied voltage was increased in order to obtain a faster analysis. However, increasing the applied voltage from -10 kV to -15 kV resulted in currents above $100\ \mu\text{A}$ which led to a poor peak resolution due to Joule heating. Thus, the voltage was reduced to -10 kV, which resulted in currents of about $75\ \mu\text{A}$ and acceptable peak shapes.

In summary, the optimized background electrolyte consisted of a 50 mM phosphate buffer, pH 3.0, containing 80 mg/mL of SBE(V)- β -CD and 25 mg/mL of sulfated β -CD. The separation was carried out in a 50 μ m id fused-silica capillary with an effective length of 35 cm (a total length of 40.2 cm) at an applied voltage of -10 kV and operated at capillary temperature 20°C . Detection was carried out at 200 nm. The separation of the enantiomers of amphetamine, the positively charged potential impurities 1*S*,2*S*(+)-norpseudoephedrine and 1*R*,2*S*(-)-norephedrine, the neutral potential impurities phenylacetone and phenylacetone oxime as well as 1*R*,2*S*(-)-ephedrine (selected as internal standard) employing the optimized conditions is shown in Fig. 5B.

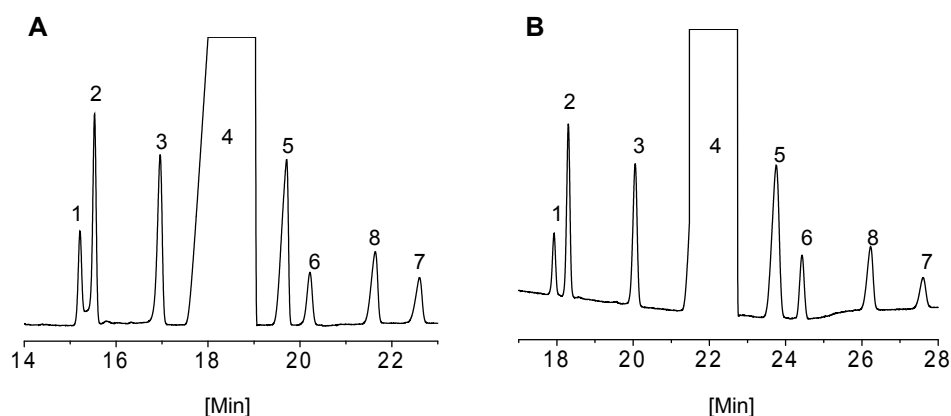


Figure 5. Effect of the pH of the background electrolyte on analyte separation of a sample of 5 mg/mL dexamphetamine sulfate containing about 3 % levoamphetamine and spiked with 1.0% (50 μ g/mL) of the impurities as well as 70 μ g/mL ephedrine (IS). Background electrolyte: 50 mM sodium phosphate buffer (A) pH 2.5, (B) pH 3.0, containing 80 mg/mL SBE(V)- β -CD and 25 mg/mL sulfated β -CD. Experimental conditions: 40.2/35 cm, 50 μ m id fused-silica capillary, -10 kV, 20°C . 1, phenylacetone Z-oxime; 2, phenylacetone E-oxime; 3, phenylacetone; 4, dexamphetamine; 5, levoamphetamine; 6, 1*S*,2*S*(+)-norpseudoephedrine; 7, 1*R*,2*S*(-)-norephedrine; 8, 1*R*,2*S*(-)-ephedrine.

Commercial sulfobutylether β -CD exists in qualities with different substitution patterns, which can affect the stereoisomer separations [69, 73]. Thus, CDs with different degree of substitution (DS), *i. e.* SBE(IV)- β -CD (DS 4) and SBE(VII)- β -CD (DS 7), were investigated in comparison to SBE(V)- β -CD. Sample containing 5 mg/mL of dexamphetamine sulfate as well as 1% of the potential impurities was analyzed. The separations obtained from the three sulfobutylether β -CDs at a concentration of 80 mg/mL and 20 mg/mL of sulfated β -CD under otherwise identical conditions were summarized in Fig. 6. The DS affected peak shape and peak resolution, especially the separation of the amphetamine enantiomers. Peak distortion was observed for SBE(IV)- β -CD (Fig. 6A), whereas peak resolution between the amphetamine enantiomers was decreased using SBE(VII)- β -CD as chiral selector (Fig. 6B). Good resolution of all analytes was obtained for SBE(V)- β -CD, but peak splitting of levoamphetamine which migrated after dexamphetamine was observed as well (Fig. 6C). Peak splitting could be overcome by increasing the concentration of sulfated β -CD to 25 mg/mL (Fig. 6D). It was noted that a further increase in a concentration of sulfated β -CD to 30 mg/mL resulted in a partial overlap of the peaks of phenylacetone and

dexamphetamine. Minor changes of the concentrations of both CDs in the range of ± 1 -2 mg/mL within the optimized conditions did not show significant effects on peak resolution or peak shape of all analytes. The concentration of the individual CDs and the composition of the BGE affect peak shape and resolution (see also Fig. 6). Therefore, the system can be optimized by adjustments of the concentration of one or both of the employed CDs as well as the buffer pH in order to achieve the sufficient peak resolution and peak shape for quantitation of all impurities. Moreover, a system suitability test using dexamphetamine and levoamphetamine with strongly different concentrations, e. g., 5 mg/mL of dexamphetamine and 3-5% of levoamphetamine, as a critical pair should be employed when sulfobutylether β -CDs with a DS different from 5 is utilized.

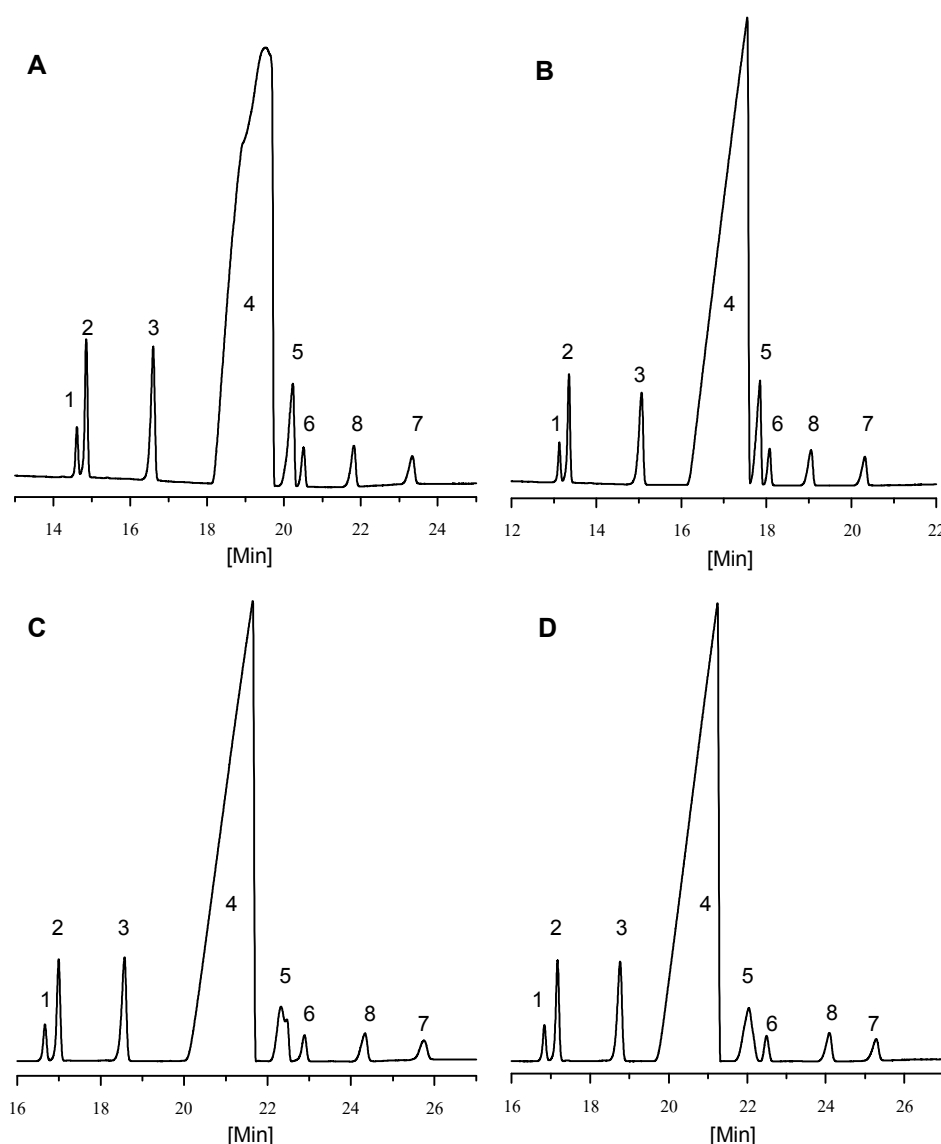


Figure 6. Effect of the DS of SBE- β -CD on peak shape and peak resolution. (A) SBE(IV)- β -CD, (B) SBE(VII)- β -CD, (C) SBE(V)- β -CD. A sample of 5 mg/mL dexamphetamine sulfate containing about 3 % levoamphetamine and spiked with 1% (50 μ g/mL) of the impurities as well as 70 μ g/mL ephedrine. Background electrolyte: 50 mM phosphate buffer, pH 3.0, containing (A) 80 mg/mL SBE(IV)- β -CD and 20 mg/mL sulfated β -CD, (B) 80 mg/mL SBE(VII)- β -CD and 20 mg/mL sulfated β -CD, (C) 80 mg/mL SBE(V)- β -CD and 20 mg/mL sulfated β -CD, and (D) 80 mg/mL SBE(V)- β -CD and 25 mg/mL sulfated β -CD. Other experimental conditions and peak assignment see Fig. 5.

The optimized method was subsequently validated according to the ICH guideline “Validation of analytical procedures”, Q2(R1) [52], with regard to linearity, range, limit of detection (LOD), limit of quantitation (LOQ) as well as intraday and interday precision. As before, method validation was performed using the racemic amphetamine. Levoamphetamine was validated over the range of 0.05-5% (2.5-250 µg/mL) based on the concentration of 5 mg/mL of dexamphetamine sulfate. All other impurities were validated over the range of 0.05-1% (2.5-50 µg/mL). 1*R*,2*S*-(–)-ephedrine was used as internal standard at a concentration of 70 µg/mL. The LOQ estimated at a signal-to-noise ratio of 10 was 0.05% (2.5 µg/mL) for all potential impurities. The LOD estimated at a signal-to-noise ratio of 3 was 0.02% (1 µg/mL) for the charged impurities and 0.01% (0.5 µg/mL) for neutral impurities. Correlation coefficients of at least 0.993 were observed indicating sufficient linearity in the investigated concentration range. The RSD values of intraday and interday precision at high and low concentrations were below 8% for all impurities indicating a sufficient repeatability of the method.

The validated method was subsequently applied for the analysis of commercial samples of dexamphetamine sulfate. Samples containing 5 mg/mL of dexamphetamine drug substance and 70 µg/mL of ephedrine were analyzed. In accordance with earlier results (Manuscript I), neither 1*R*,2*S*-(–)-norephedrine nor 1*S*,2*S*-(+)-norpseudoephedrine could be detected in any sample. In contrast, levoamphetamine was present at concentrations between 3.2 and 3.7%, which are in accordance with the earlier study as well. Interestingly, peaks corresponding to phenylacetone and isomers of phenylacetone oxime were observed at a concentration of the LOD or below. Regarding the disregard limit of the European Pharmacopoeia [55] the neutral impurity with quantities below the LOQ would not be considered necessary. However, the presence of phenylacetone and the oximes demonstrate that the investigated batches of commercial dexamphetamine sulfate were synthesized starting from phenylacetone via the oxime and subsequently resolved the enantiomers by fractional crystallization. The analysis of commercial dexamphetamine sulfate samples is summarized in Table 2. The quantities of levoamphetamine obtained from peak area normalization are generally higher but are in general agreement with the values obtained from the calibration data.

Table 2. Impurity profile of commercial dexamphetamine sulfate samples (mean ± SD, n = 3)

Compound	Sample 1	Sample 2	Sample 3	Sample 4
Phenylacetone <i>Z</i> -oxime	ND ^{a)}	LOD ^{b)}	LOD	LOD
Phenylacetone <i>E</i> -oxime	LOD	LOD	LOD	LOD
Phenylacetone	LOD	LOD	LOD	LOD
1 <i>S</i> ,2 <i>S</i> -(+)-Norpseudoephedrine	ND	ND	ND	ND
1 <i>R</i> ,2 <i>S</i> -(–)-Norephedrine	ND	ND	ND	ND
Levoamphetamine (calibration)	3.23 ± 0.02	3.70 ± 0.15	3.38 ± 0.09	3.36 ± 0.06
Levoamphetamine (normalization)	3.88 ± 0.06	4.25 ± 0.07	4.01 ± 0.11	3.47 ± 0.04

a) ND: not detectable

b) LOD: detected at the LOD level

3.1.3 Impurity profiling of dexamphetamine sulfate by cyclodextrin-modified microemulsion electrokinetic chromatography (Manuscript III)

Microemulsion electrokinetic chromatography (MEEKC) has proven to be a powerful technique for the analysis of charged and uncharged compounds including stereoisomer analysis. However, since the application of MEEKC to the simultaneous determination of achiral impurities of drugs and the analysis of the enantiomeric purity has not been reported to date, the present study was conducted in order to evaluate cyclodextrin-modified MEEKC for the simultaneous determination of stereoisomeric purity as well as impurity profiling of dexamphetamine sulfate. The drug and its impurities (Fig. 7) comprise charged and uncharged analytes, chiral and achiral analytes or a pair of enantiomers as well as a pair of *E*- and *Z*-geometrical isomers. Dexamphetamine and levoamphetamine represent the pair of enantiomers as well as charged compounds. Phenylacetone is uncharged and achiral compound. Phenylacetone oxime represents uncharged compound as well as the pair of geometric isomers. Both 1*S*,2*S*-(+)-norpseudoephedrine and 1*R*,2*S*-(-)-norephedrine represent charged and chiral compounds.

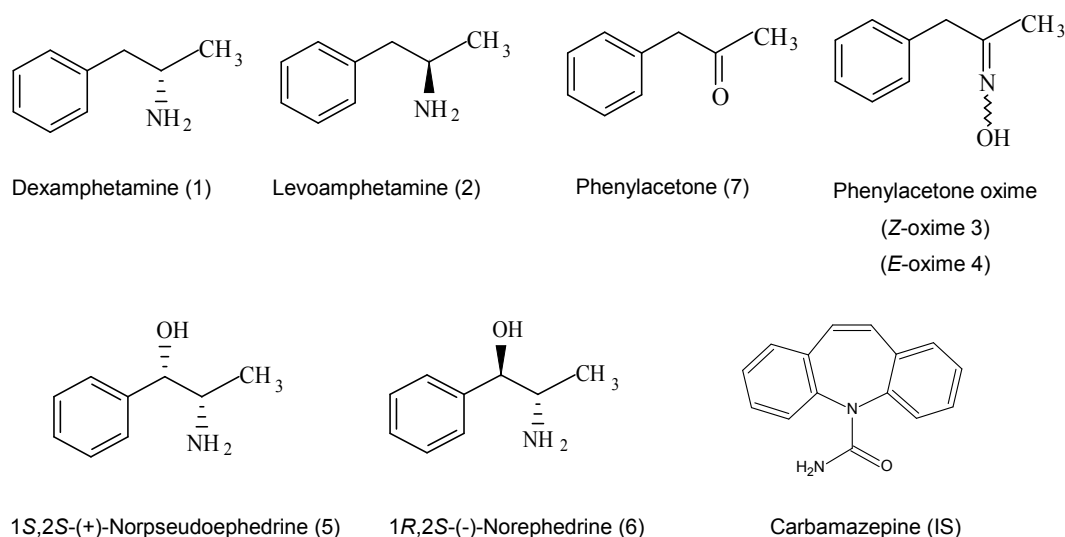


Figure 7. Structures of dexamphetamine and potential impurities as well as the internal standard carbamazepine.

Micellar electrokinetic chromatography (MEKC) was initially evaluated using background electrolytes with pH values of 9.5 and 3.0 containing 3.5% w/w SDS and 5-8% of organic solvent such as 1-butanol, 2-propanol, acetonitrile or methanol. None of the investigated conditions resulted in acceptable peak shape, baseline noise or (achiral) separation of all analytes (see also Fig. 8). However, as the initial test employing 2-propanol modified MEKC at low pH with reversed polarity of the applied voltage showed a promising baseline separation of amphetamine and neutral compounds, MEEKC employing a BGE at a low pH was further considered.

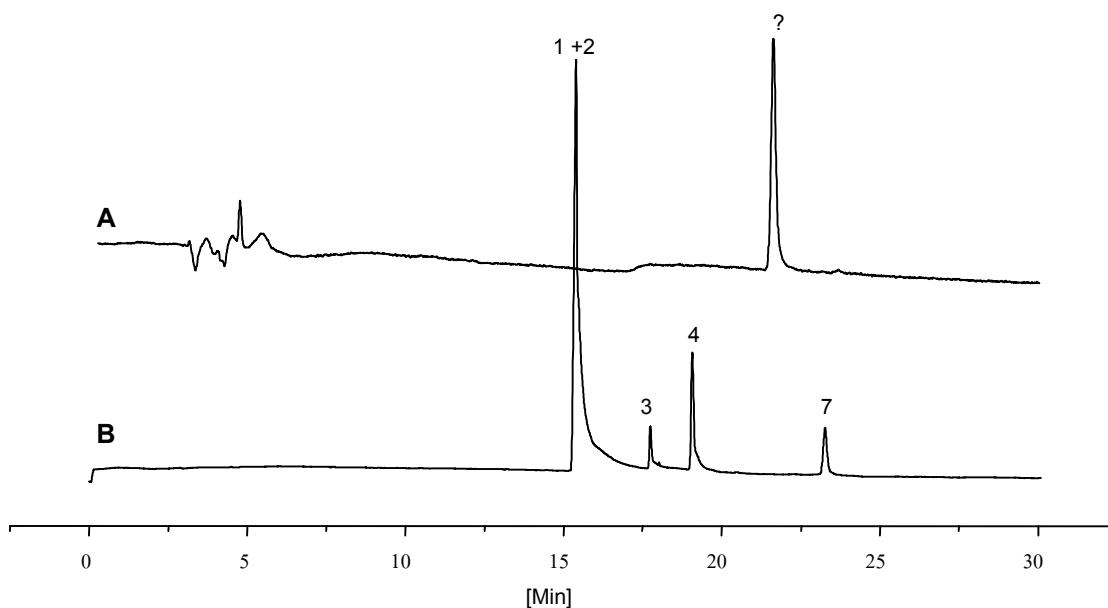


Figure 8. The electropherograms of sample containing 1 mg/mL of racemic amphetamine, 0.2 mg/mL of phenylacetone oxime and 0.1 mg/mL of phenylacetone. The BGE consisted of (A) 5 mM sodium borate buffer, pH 9.5 (normal polarity); (B) 50 mM sodium phosphate buffer, pH 3.0 (reversed polarity), containing 3.5% w/w SDS and 8% w/w 2-propanol. For peak assignments, see Fig. 7.

Initially, tested MEEKC conditions were modified starting from a suppressed electroosmosis CD-modified MEEKC assay for the enantioseparation of phenylalanine derivatives published by Brost and Holzgrabe [74]. Thus, a microemulsion containing a 50 mM phosphate buffer, pH 3.0, ethyl acetate, SDS, 1-butanol, 2-propanol and sulfated β -CD was evaluated for the simultaneous determination of dexamphetamine and the *R*-enantiomer as well as related substances. A solution of 2 mg/mL dexamphetamine sulfate containing about 3% of levoamphetamine and 1% of the impurities as well as 1*R*,2*S*-(–)-ephedrine as internal standard was used for method development. Fig. 9A shows the separation of the analytes using a microemulsion composed of 1.4% w/w SDS, 1.0% w/w ethyl acetate, 4.0% w/w 1-butanol, 2.8% w/w 2-propanol, 90.8% w/w 50 mM phosphate buffer, pH 3.0, containing 4.0% w/w sulfated β -CD under reversed polarity of the applied voltage. Although the enantiomers of amphetamine were well separated, the intended internal standard 1*R*,2*S*-(–)-ephedrine comigrated with 1*S*,2*S*-(+)-norpseudoephedrine and phenylacetone *Z*-oxime was not completely separated from dexamphetamine. It was noted that the separation of all analytes could be resolved by variations of the composition of the microemulsion and the CD concentration. Thus, the effects of each composition in the background electrolyte on the separation were investigated and discussed as follows.

- *SDS (the surfactant)*: Increasing the concentration of SDS to 2.0% w/w essentially led to a loss of the enantioseparation of amphetamine and a partial overlap of 1*R*,2*S*-(–)-ephedrine, 1*S*,2*S*-(+)-norpseudoephedrine and phenylacetone *E*-oxime. Thus, the concentration of 1.5% w/w SDS was selected for further method development.

- *Ethyl acetate (the oil core)*: It was observed that the content of ethyl acetate used as an oil core had a significant effect to the separation of all analytes, especially for the separation of the

amphetamine enantiomers. Decreasing the content of ethyl acetate to 0.5% w/w with a reduction of the co-surfactant 1-butanol to 3.0% w/w and the organic modifier 2-propanol to 2.0% w/w resulted in a comigration of the amphetamine enantiomers, although all other analytes were sufficiently separated (Fig. 9B). Increasing the content of ethyl acetate to 1.0% w/w resulted in an improvement of the enantioseparation, but the partial overlap of 1*S*,2*S*-(+)-norpseudoephedrine and 1*R*,2*S*-(–)-norephedrine was observed as well (Fig. 9C). Because of the baseline separation of related compounds, the content of ethyl acetate at 0.5% w/w was selected for further optimization of the CD concentration in order to improve the baseline separation of dexamphetamine and levoamphetamine.

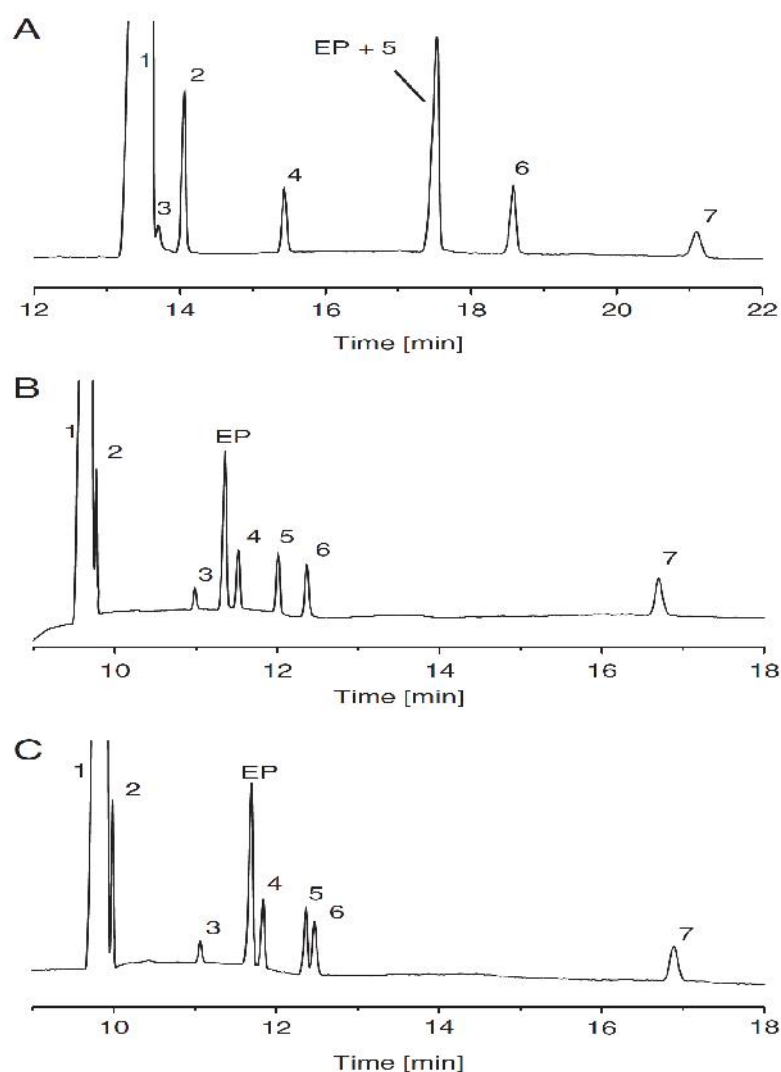


Figure 9. Effect of the composition of the microemulsion on analyte separation. Background electrolyte: (A) 1.4% w/w SDS, 1.0% w/w ethyl acetate, 4.0% w/w 1-butanol, 2.8% w/w 2-propanol, 90.8% w/w phosphate buffer, pH 3.0, containing 4.0% w/w sulfated β -CD; (B) 1.5% w/w SDS, 0.5% w/w ethyl acetate, 3.0% w/w 1-butanol, 2.0% w/w 2-propanol, 93.0% w/w phosphate buffer, pH 3.0, containing 4.0% w/w sulfated β -CD; (C) 1.5% w/w SDS, 1.0% w/w ethyl acetate, 3.0% w/w 1-butanol, 2.0% w/w 2-propanol, 92.5% w/w phosphate buffer, pH 3.0, containing 4.0% w/w sulfated β -CD. Other experimental conditions: 50.2/40 cm, 50 μ m id fused-silica capillary, -15 kV, 20°C; sample: 2 mg/mL dexamphetamine sulfate containing about 3% levoamphetamine and 1% of the other impurities as well as 70 μ g/mL 1*R*,2*S*-(–)-ephedrine. For peak assignment, see Fig. 7.

- *Sulfated β -CD (the chiral selector)*: The CD was intended as a chiral selector for the amphetamine enantiomers. Subsequently, the variation of the CD concentration from 4% w/w to 8% w/w was investigated (see also Fig. 10). As partial overlap of phenylacetone *E*-oxime and 1*R*,2*S*-(-)-ephedrine was observed when a CD concentration above 5% w/w was used, 1*R*,2*S*-(-)-ephedrine was not further employed. Instead, carbamazepine was selected as an internal standard due to the peak shape and short migration time. Baseline separation of all analytes could be achieved by increasing the concentration of sulfated β -CD to 5.5% w/w (Fig. 8). A further increase of sulfated β -CD above 6% w/w resulted in unstable baseline as well as partial overlap of phenylacetone *Z*-oxime and dexamphetamine. Thus, sulfated β -CD at a concentration of 5.5% w/w was used for further optimization.

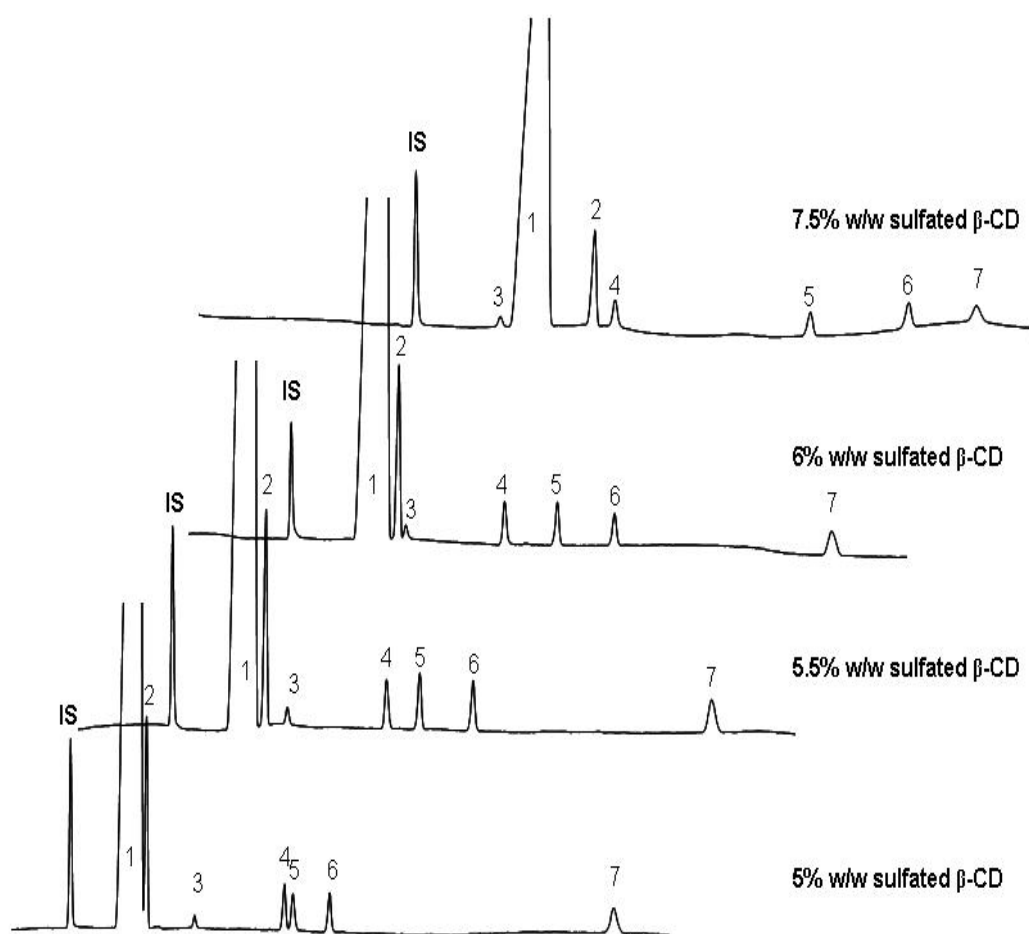


Figure 10. Effects of CD concentration on analyte separation. Background electrolyte: 1.5% w/w SDS, 0.5% w/w ethyl acetate, 3.0% w/w 1-butanol, 2.0% w/w 2-propanol, 93.0% w/w phosphate buffer, pH 3.0, containing concentrations between 5.0 and 7.5% w/w of sulfated β -CD. Sample: 2 mg/mL dexamphetamine sulfate containing about 3% levoamphetamine and 1% of the other impurities as well as 50 μ g/mL carbamazepine. For other experimental conditions, see Fig. 9. For peak assignment, see Fig. 7.

- *1-Butanol and 2-propanol (co-surfactant and organic modifier)*: It was observed that an increase in the content of 1-butanol or 2-propanol in the BGE resulted in an improvement of the enantioseparation of amphetamine. Therefore, the final fine tuning of the microemulsion was performed by variation of the contents of 1-butanol between 3.0 and 4.0% w/w and of 2-propanol in the range between 2.0 and 3.0% w/w. The best separation of all analytes was achieved using 3.5% w/w 1-butanol and 2.5% w/w 2-propanol, which allowed the separation of a sample of 3 mg/mL dexamphetamine sulfate. A further increase in 1-butanol and 2-propanol above 4% w/w and 3% w/w, respectively, may lead to a disruption of microemulsion system, since none of impurities could be detected and an unstable baseline was observed as well.

- *Buffer concentration and buffer pH*: Furthermore, the buffer concentration was varied between 20 and 100 mM. Reduction of the buffer concentration to 20 mM resulted in peak broadening, while an increase in the buffer pH to 100 mM led to currents above 100 μ A. Decreasing the buffer pH to 2.0 led to a depletion of the peak height of phenylacetone *E*-oxime, whereas the other compounds were not affected.

The final optimized method employed the background electrolyte containing 1.5% w/w SDS, 0.5% w/w ethyl acetate, 3.5% w/w 1-butanol, 2.5% w/w 2-propanol and 92% w/w 50 mM phosphate buffer, pH 3.0, containing 5.5% w/w sulfated β -CD. The separation was carried out in a 50 μ m id fused-silica capillary with an effective length of 40 cm at an applied voltage of -14 kV (currents between 73 and 78 μ A), operated at 20°C. The detection wavelength was 200 nm. The separation of a sample of 3 mg/mL dexamphetamine sulfate containing about 3% levoamphetamine, 1% of the impurities and 50 μ g/mL of carbamazepine as internal standard is shown in Fig. 11A.

During method development, it was observed that preparation of the microemulsion affected the separation between dexamphetamine and levoamphetamine as well as between phenylacetone *E*-oxime and 1*S*,2*S*-(+)-norpseudoephedrine depending on the addition of sulfated β -CD either before or after the preparation of the microemulsion. No apparent explanation can be concluded for this phenomenon. Good resolution between the amphetamine enantiomers as well as phenylacetone *E*-oxime and 1*S*,2*S*-(+)-norpseudoephedrine was achieved using the background electrolyte prepared by adding sulfated β -CD to the phosphate buffer prior to the preparation of the microemulsion (see also Fig. 11A). Thus, this background electrolyte was used for the validation and sample analysis. A slightly decrease in the resolution was observed employing a background electrolyte prepared by adding sulfated β -CD to the phosphate buffer after the preparation of the microemulsion (Fig. 11B).

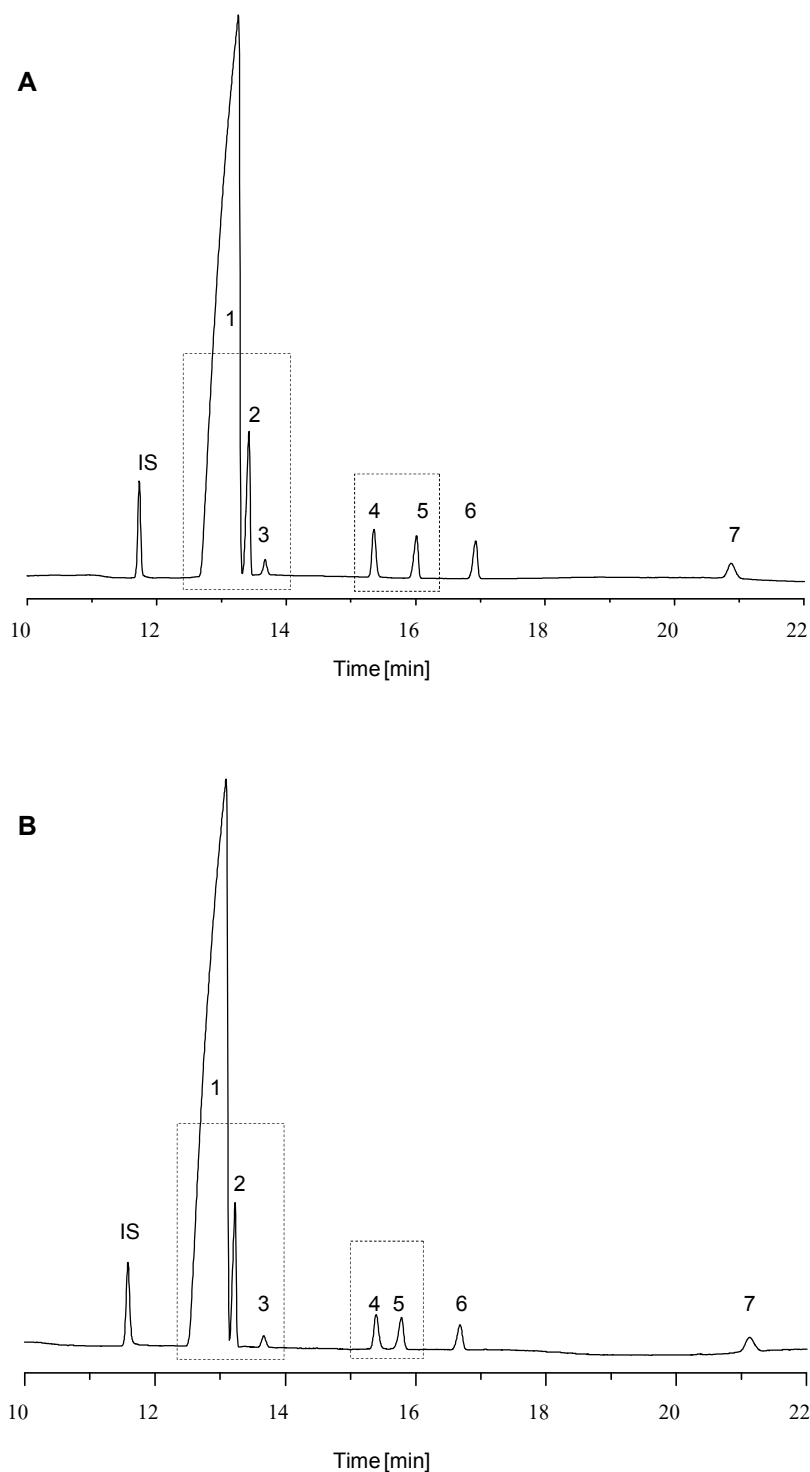


Figure 11. Effect of preparation of the background electrolyte on an analyte separation. (A) Addition of sulfated β -CD to the phosphate buffer prior to the preparation of the microemulsion by sonication and (B) addition of sulfated β -CD to the phosphate buffer after preparation of the microemulsion. Experimental conditions: 50.2/40 cm, 50 μ m id fused-silica capillary, -15 kV, 20°C ; background electrolyte: 1.5% w/w SDS, 0.5% w/w ethyl acetate, 3.5% w/w 1-butanol, 2.5% w/w 2-propanol and 92% w/w 50 mM phosphate buffer, pH 3.0, containing 5.5% w/w sulfated β -CD; sample: 3 mg/mL dexamphetamine sulfate containing about 3% levoamphetamine and 30 $\mu\text{g/mL}$ (1%) of the other impurities as well as 50 $\mu\text{g/mL}$ carbamazepine as internal standard (IS). For peak assignment, see Fig. 7.

The optimized method was subsequently validated according to the ICH guideline "Validation of analytical procedures", Q2(R1). Method validation was performed using racemic amphetamine. Levoamphetamine was validated over the range of 3-150 $\mu\text{g/mL}$ corresponding to 0.1-5% based on the concentration of 3 mg/mL of dexamphetamine sulfate. All other impurities were validated over the range of 3-30 $\mu\text{g/mL}$ (0.1-1%) except for phenylacetone Z-oxime which was calibrated between 15 and 30 $\mu\text{g/mL}$ (0.5-1%). Carbamazepine was used as internal standard at a concentration of 50 $\mu\text{g/mL}$. The LOQ estimated at a signal-to-noise ratio of 10 was 0.1% (3 $\mu\text{g/mL}$) for all potential compounds except for phenylacetone Z-oxime with a LOQ of 0.5% (15 $\mu\text{g/mL}$). The LOD estimated at a signal-to-noise ratio of 3 was 0.05% (1.5 $\mu\text{g/mL}$) for the impurities, while a LOD for the Z-oxime was 0.2% (6 $\mu\text{g/mL}$). Correlation coefficients of at least 0.994 were observed indicating sufficient linearity in the investigated concentration range. The repeatability of the method was expressed by the relative standard deviation (RSD) values of the peak area ratios between the analyte and the internal standard. The RSD values of intraday and interday precision at high and low concentrations were below 8.2% for all impurities.

Four commercial samples of dexamphetamine sulfate were subsequently analyzed employing the validated method. Levoamphetamine was detected in all dexamphetamine samples at concentrations between 3.22 ± 0.10 and $3.82 \pm 0.07\%$ ($n=3$). Other potential impurities could not be detected because the employed CD-modified MEEKC method is less sensitive than the method employing a dual CD system.

3.1.4 Concluding remarks (Manuscript I, II and III)

Three stereoselective CE assays were developed and validated for the simultaneous determination of dexamphetamine and levoamphetamine as well as related substances. A CE assay employing HDAS- β -CD as a chiral selector was initially developed. The assay was subsequently applied for the determination of the stereoisomeric purity of commercial dexamphetamine samples. As none of the charged impurities 1S,2S-(+)-norpseudoephedrine and 1R,2S-(−)-norephedrine could be detected, a stereoselective CE assay employing a dual CD system comprising of SBE(V)- β -CD and sulfated β -CD was subsequently developed. The CE assay employing the dual CD system provides a lower LOQ value than the method using a single CD system, moreover the amphetamine enantiomers as well as potential charged, 1S,2S-(+)-norpseudoephedrine and 1R,2S-(−)-norephedrine and neutral impurities, phenylacetone and phenylacetone oxime, can be detected simultaneously. Subsequently, a CE assay employing CD-modified MEEKC was developed as an alternative method to the use of the expensive CDs HDAS- β -CD and sulfobutylether β -CD. The optimized CD-modified MEEKC method allowed the simultaneous determination of the amphetamine enantiomers as well as potential charged and uncharged related substances. Among those developed CE assays, the dual CD system seems to be the most suitable analytical method for the determination of dexamphetamine and related substances. Both charged and uncharged compounds can be analyzed simultaneously and the impurities can be quantified at a lower LOQ value compared to the CE assays employing either a single CD system or CD-modified MEEKC technique. A disadvantage is the fact that SBE(V)- β -CD is rather

expensive. Finally, three different CE assays used for the simultaneous determination of the enantiomeric purity of dexamphetamine sulfate and its potential impurities are summarized and compared in Table 3.

Table 3. Comparison of stereoselective CE assays for the simultaneous determination of the enantiomeric purity of dexamphetamine sulfate and related substances.

CE assays	Single CD system	Dual CD system	CD-modified MEEKC
Optimized method:			
Chiral selector	HDAS- β -CD	SBE(V)- β -CD and sulfated β -CD	sulfated β -CD
Buffer	100 mM phosphate buffer, pH 2.5	50 mM phosphate buffer, pH 3.0	microemulsion in 50 mM phosphate buffer, pH 3.0
Range	0.06 – 5.0%	0.05 – 1.0% 0.05 – 5.0% (R-Amp)	0.1 – 1.0%, 0.5 – 1.0% (Z-oxime) 0.1 – 5.0% (R-Amp)
LOQ	0.06%	0.05%	0.1% 0.5% (Z-oxime)
LOD	0.02% 0.03% (R-Amp)	0.01% (neutral cpd.) 0.02% (charged cpd.)	0.05% 0.2% (Z-oxime)
Correlation coefficient (r)	>0.995	>0.993	>0.994
Precision (%RSD)	<7%	<8%	<8.2%
Sample analysis	2 mg/mL	5 mg/mL	3 mg/mL
Internal standard	1 <i>R</i> ,2 <i>S</i> -(-)-ephedrine	1 <i>R</i> ,2 <i>S</i> -(-)-ephedrine	carbamazepine
Analysis time	15 min	30 min	22 min
Determination	Only charged compounds	Charged and neutral compounds	Charged and neutral compounds
Cost analysis:			
Chiral selector	HDAS- β -CD; <i>expensive</i>	SBE(V)- β -CD; <i>expensive</i> sulfated β -CD; <i>relatively inexpensive</i>	sulfated β -CD; <i>relatively inexpensive</i>
Organic solvents and surfactant	Not applicable	Not applicable	SDS; 166 \$ /kg EtOAc; 81.82 \$ /L 1-BuOH; 61.3 \$ /0.5L 2-PrOH; 39.76 \$ /0.5L

SDS; sodium dodecyl sulfate, EtOAc; ethyl acetate, 1-BuOH; 1-butanol, 2-PrOH; 2-propanol

3.2 A stereoselective CE assay for the simultaneous determination of related substances and the enantiomeric purity of levodopa: comparison with the pharmacopeial polarimetric and HPLC methods (Manuscript IV)

Levodopa is the (S)-enantiomer of 3,4-dihydroxyphenylalanine (Fig. 12) that is used in the treatment of Parkinson's disease. Levodopa and tests for the determination of related substances and of the enantiomeric purity are described in the European Pharmacopoeia [55] and the United States Pharmacopeia [53]. The related substances specified by the pharmacopeias include L-tyrosine (Tyr), 3-methoxy-L-tyrosine (3-OMe-Tyr), and racemic 6-hydroxy-D,L-dopa (6-OH-DOPA) as shown in Fig 12. As no assay allowing the simultaneous determination of enantiomeric purity of levodopa as well as related substances has been published, a stereoselective CE assay was developed.

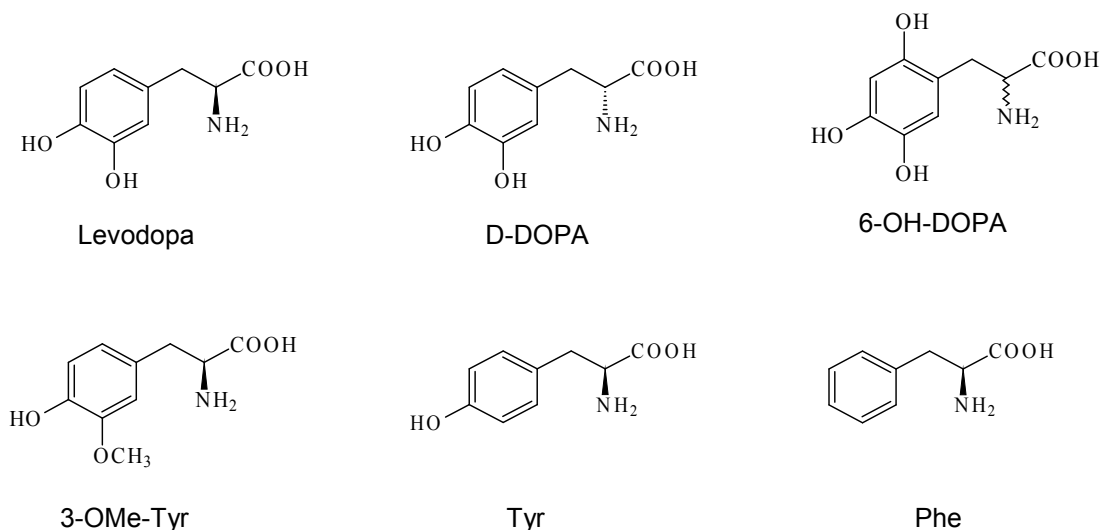


Figure 12. Structures of levodopa, D-DOPA and related substances as well as the internal standard L-Phe.

Initial screening tests employed a sodium phosphate buffer containing 2 mg/mL sulfated β -CD with normal polarity of the applied voltage. The buffer molarity between 20 mM and 200 mM, pH between 2.0 and 3.0, was evaluated in a capillary with an effective length of 40 cm and an applied voltage of 20 kV. Sharp peaks and short migration times were observed lowering the buffer pH from 3.0 to 2.0. With regard to buffer molarity, a 0.12 M phosphate buffer provided a good compromise between peak shape and electric current (below 100 μ A). However, under these conditions Tyr comigrated with levodopa. Subsequently, the CD concentration was varied between 2 and 8 mg/mL in order to achieve baseline separation and acceptable peak shape of all analytes (see also Fig. 13). At a concentration of 8 mg/mL of sulfated β -CD good resolutions as well as sharp peaks were achieved for all compounds. In addition, the stereoisomers of 6-OH-DOPA were separated at a CD concentration of 4 mg/mL or above.

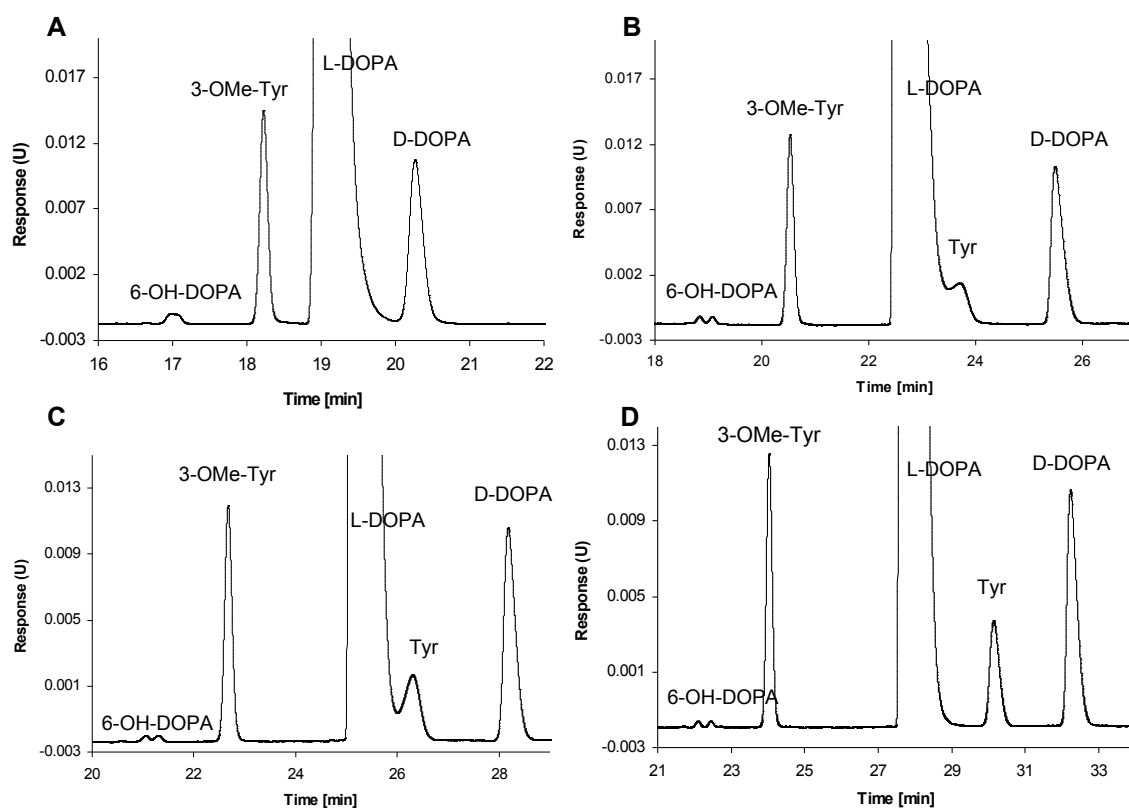


Figure 13. Effect of the concentration of sulfated β -CD on the separation of levodopa and its specified impurities. Experimental conditions: 0.12 M phosphate buffer, pH 2.0, containing (A) 2 mg/mL sulfated β -CD, (B) 4 mg/mL sulfated β -CD, (C) 6 mg/mL sulfated β -CD, and (D) 8 mg/mL sulfated β -CD; 50.2/40 cm fused-silica capillary, 20 kV, 18°C. Sample concentration: 2 mg/mL levodopa containing 1.0% of 3-OMe-Tyr and D-DOPA, 0.5% of Tyr, and 0.1% of 6-OH-DOPA.

Interestingly, when the phosphate buffer was prepared from orthophosphoric acid and sodium hydroxide, the buffer molarity and sulfated β -CD could be reduced to 0.1 M and 6 mg/mL, respectively, with comparable resolution as shown in Fig. 13D. Increasing the applied voltage to 25 kV led to the currents above 100 μ A, moreover the partial overlap between Tyr and D-DOPA was observed. Thus, the optimized conditions employed a 0.1 M phosphate buffer, pH 2.0, prepared from orthophosphoric acid and sodium hydroxide solution containing 6 mg/mL of sulfated β -CD as background electrolyte, a 50 μ m id fused-silica capillary with an effective length of 35 cm (total length 45.2 cm), an applied voltage of +20 kV and 18°C capillary temperature. Due to the fact that a randomly substituted sulfated β -CD as commercially available and low price CD derivative was selected in the present study, two batches of the CDs from other suppliers were tested with regard to the reproducibility of the separation of the analytes. Clearly, the different samples of sulfated β -CD affected the separation, especially the critical resolution between levodopa and Tyr. However, an increase in the CD concentration can improve the resolution of the analytes.

The optimized method was subsequently validated according to the ICH guideline "Validation of analytical procedures", Q2(R1). All impurities were validated over the range of 2-20 μ g/mL corresponding to 0.1-1% based on the concentration of 2 mg/mL of levodopa drug substance. L-Phenylalanine was used as internal standard at a concentration of 20 μ g/mL. The LOQ estimated

at a signal-to-noise ratio of 10 was 0.1% (2 µg/mL) for all impurities. The LOD estimated at a signal-to-noise ratio of 3 was between 0.03% (0.6 µg/mL) and 0.04% (0.8 µg/mL). Correlation coefficients of at least 0.990 were observed indicating sufficient linearity in the investigated concentration range. The RSD values of intraday and interday precision at high and low concentrations were below 10% for all impurities.

The method was subsequently applied to determine the related substances as well as D-DOPA in samples of levodopa including the chemical reference substance of the European Pharmacopoeia. Neither 6-OH-DOPA nor 3-OMe-Tyr could be detected. In contrast, Tyr could be detected in all samples at a concentration below the LOQ, which is in accordance with the data reported by Schieffer G. W. [75]. An amount of D-DOPA of $0.15 \pm 0.01\%$ was obtained from the reference substance of the European Pharmacopoeia, which is in accordance with the requirement of the European Pharmacopoeia for enantiomeric purity test of levodopa.

The related substances of levodopa are determined by comparable HPLC assays by the European Pharmacopoeia and the United States Pharmacopeia. The enantiomeric purity of levodopa is determined by optical rotation by the United States Pharmacopeia based on the increased optical rotation of levodopa in the presence of methenamine while the European Pharmacopoeia uses enantioselective HPLC based on a chiral ligand exchange method published by Doležalová and Tkaczyková [76]. A LOD of D-DOPA of 0.04% was reported, which is more sensitive than the measurement of the optical rotation of the sample. However, the negative peaks are observed between the peak of levodopa and D-DOPA, which may make reproducible integration difficult. The electropherogram of a sample containing 2 mg/mL levodopa and 0.5 % of the impurities using the present CE assay and a chromatogram of HPLC assay are shown in Fig. 14. Furthermore, a comparison made for the official analytical methods and the present CE assay regarding the determination of the enantiomeric purity of levodopa and the related substances is shown in Table 4. The simultaneous determination of enantiomeric purity and related substances of levodopa as specified by the European Pharmacopoeia can be achieved only by employing the present CE assay, which also provides a sufficient sensitivity with a LOQ of 0.1% for all impurities as well as short analysis time for the determination of all analytes. Moreover, since the resolution of all analytes as shown in Fig. 14B is rather, a high increase in a sample amount of major compound along with or without an increase in a CD concentration in order to obtain a LOQ value below the 0.1% level may be possible.

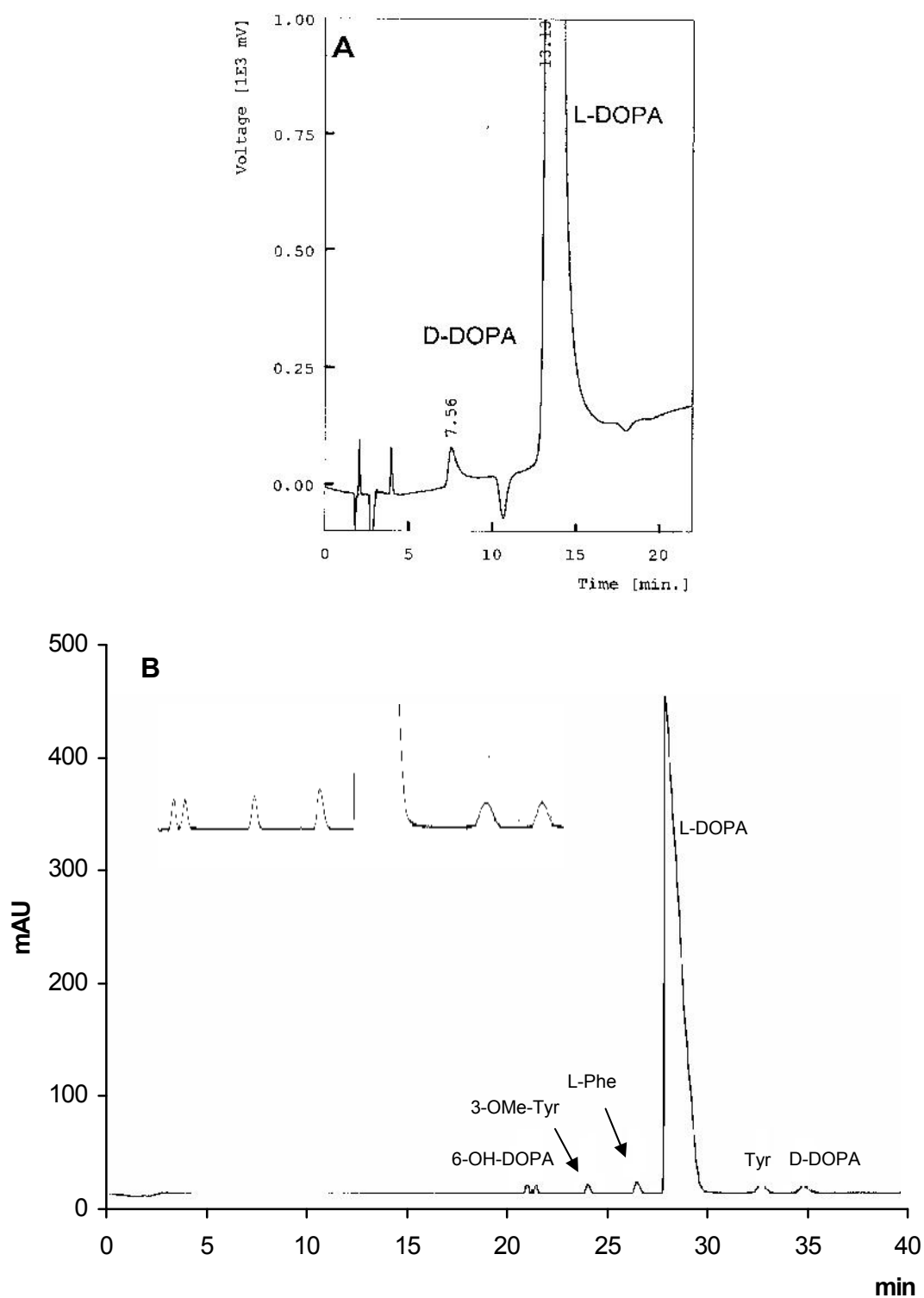


Figure 14. (A) Chromatogram of levodopa containing 0.5% of D-DOPA (reproduced from Ref. [76]) and (B) Electropherogram of 2 mg/mL levodopa containing 0.5% of D-DOPA as well as related substances and 20 μ g/mL of L-Phe as internal standard.

Table 4. Official analytical methods and the stereoselective CE assay used for the determination of the enantiomeric purity and related compounds of levodopa drug substance.

	USP 32 ^{a)}	Ph. Eur ^{b)}	The present CE
Enantiomeric purity:			
Method	Polarimetric	HPLC (ligand exchange)	CD-mediated CZE
Experimental conditions	specific rotation of levodopa in the presence of methenamine	<i>Column</i> ; 3.9 mm × 15 cm, spherical end-capped octadecylsilyl silica gel <i>Mobile phase</i> ; copper acetate and N,N-dimethyl-L-phenylalanine in water, pH 4.0, and methanol Flow rate; 1.0 mL/min	<i>Capillary</i> ; 45.2/35 cm, 50 µm id fused-silica capillary, 20 kV, 18°C <i>BGE</i> : 0.1 M phosphate buffer, pH 2.0, containing 6 mg/mL sulfated β-CD
Analysis time	at least 3 hours	20 min	35 min
LOD	Not available	0.04% ^{c)}	0.04%
Related substances:			
Method	HPLC	HPLC (ligand exchange)	CD-mediated CZE
Experimental conditions	<i>Column</i> ; 4.6 mm × 25 cm, spherical octadecyl silane <i>Mobile phase</i> ; a mixture of trifluoroacetic acid in water and tetrahydrofuran (97:3 V/V) Flow rate; 1 mL/min	<i>Column</i> ; 4.6 mm × 25 cm, spherical di-isobutyloctadecylsilyl silica gel <i>Gradient elution</i> ; (A) 0.1 M phosphate buffer, pH 3.0, (B) methanol and 0.1 M phosphate buffer, pH 3.0 (18:85 V/V) Flow rate; 1 mL/min	<i>Capillary</i> ; 45.2/35 cm, 50 µm id fused-silica capillary, 20 kV, 18°C <i>BGE</i> : 0.1 M phosphate buffer, pH 2.0, containing 6 mg/mL sulfated β-CD
Analysis time	Not available	6 min for levodopa	35 min
LOD	Not available	Not available	0.03% - 0.04%
Comments:			
Simultaneous determination of DOPA and related cpd.	Not possible	Not possible	Possible. All compounds can be detected in one injection with total analysis time of 35 min

a) The United State Pharmacopeia (32th Edition) [53]

b) The European Pharmacopoeia (7th Edition) [55]

c) reported by Doležalová and Tkaczyková [76]

3.3 Development and validation of a capillary electrophoresis assay for the determination of the stereoisomeric purity of chloroquine enantiomers (Manuscript V)

Chloroquine is commercially available as racemate used as chemotherapeutic drug for the prophylaxis and the treatment of malaria. Differences in the antimalarial activity and toxicity of the enantiomers have been reported *in vivo*. Moreover, the enantiomers appear to be less embryotoxic compared to the racemate. Due to these data the further study of stereoselective pharmacology and pharmacodynamics of the drug is necessary. Thus, an assay for the determination of the enantiomeric purity is important. The separation of the chloroquine enantiomers has been reported using charged CD derivatives as chiral selectors in acidic background electrolytes [77–80]. Due to the commercial availability sulfated β -CD and SBE- β -CD were evaluated. Initially, a 50 mM sodium phosphate buffer, pH 2.5, containing a concentration of sulfated β -CD between 5 and 30 mg/mL was evaluated as background electrolyte. Upon applying a voltage of 15 or 20 kV under normal polarity, none of the enantiomers could be detected. In contrast, the enantiomers were detected upon reversing the polarity of the applied voltage (Fig. 15A).

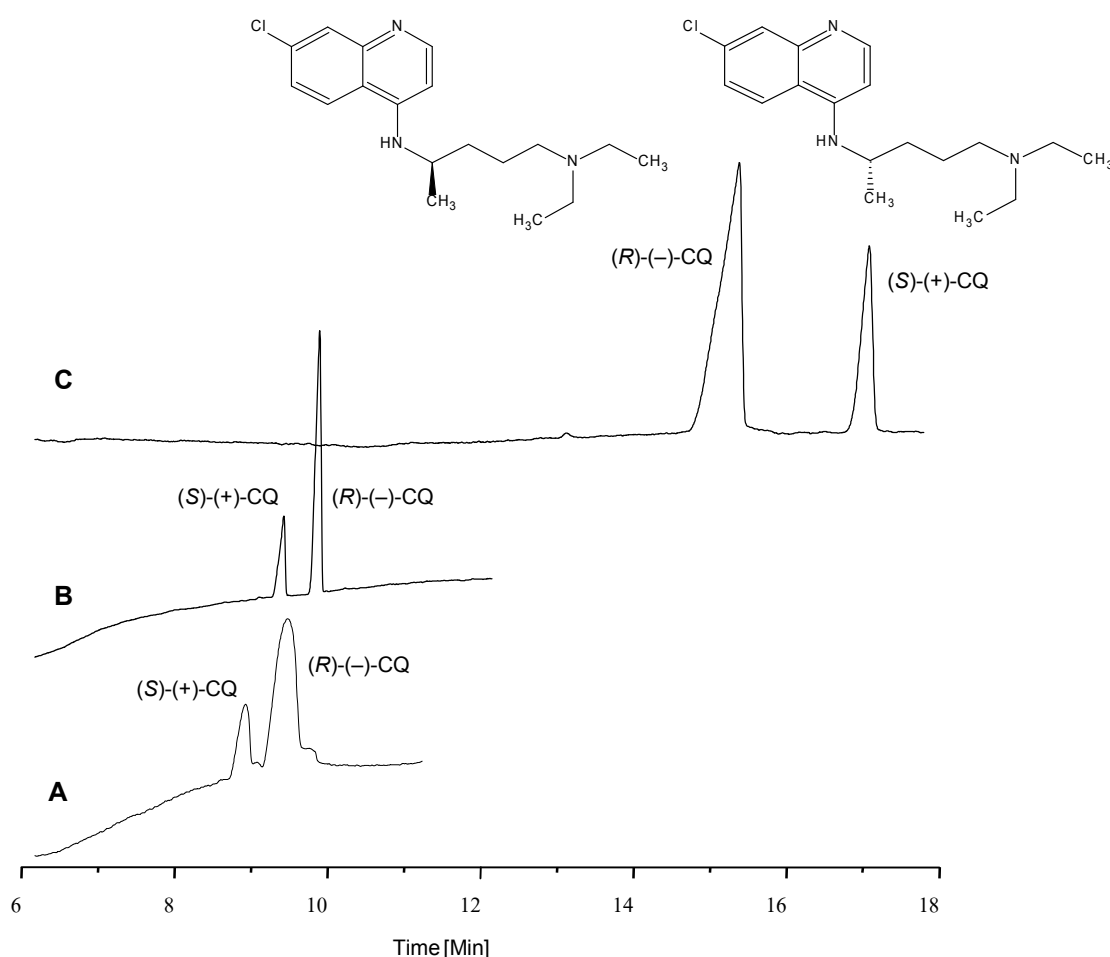


Figure 15. Electropherograms of the separation of a non-racemic mixture of chloroquine (400 μ g/mL (R)-(-)-chloroquine ((R)-(-)-CQ) and 200 μ g/mL (S)-(+)-chloroquine ((S)-(+)-CQ)) using sulfated β -CD (A and B) and SBE(VII)- β -CD (C) as chiral selector. Experimental conditions: 50.2/40 cm, 50 μ m id fused-silica capillary, 20°C, 225 nm, (A) 50 mM sodium phosphate buffer, pH 2.5, 20 mg/mL sulfated β -CD, -15 kV, (B) 100 mM sodium phosphate buffer, pH 2.5, 20 mg/mL sulfated β -CD, -15 kV and (C) 100 mM sodium phosphate buffer, pH 2.5, 20 mg/mL SBE(VII)- β -CD, -25 kV.

However, peak broadening of the enantiomers was observed. Increasing the buffer molarity resulted in sharp peaks and an improvement of the resolution (Fig. 15B). Injection of a sample containing 2 mg/mL (*R*)-(–)-chloroquine and about 20 µg/mL (*S*)-(+)-chloroquine resulted in a severely distorted peak of the minor enantiomer which could not be improved by increasing the concentration of sulfated β-CD or the buffer concentration or variation of the applied voltage. Subsequently, SBE-β-CD with degree of substitution of 5 (DS 5) and 6.6 (DS 7) was evaluated. The enantioseparation could not be achieved using SBE(V)-β-CD, whereas using SBE(VII)-β-CD and reversed polarity of the applied voltage resulted in a good resolution of the chloroquine enantiomers (Fig. 15C). Reversal of the enantiomer migration order compared to sulfated β-CD was observed.

Finally, the concentration of SBE(VII)-β-CD was increased to 30 mg/mL, which allowed the separation of a sample containing 3 mg/mL of (*R*)-(–)-chloroquine with acceptable peak shapes as well as resolution and short migration times. Thus, the optimized conditions employed a 100 mM sodium phosphate buffer, pH 2.5, containing 30 mg/mL SBE(VII)-β-CD as background electrolyte, a 50 µm id fused-silica capillary with an effective length of 40 cm, an applied voltage of –25 kV and 20°C capillary temperature. The analytes were detected at 225 nm. Carbamazepine was used as internal standard due to its sharp peak and short migration time at about 6 min.

The optimized method was subsequently validated according to the ICH guideline “Validation of analytical procedures”, Q2(R1). Method validation was performed using the chloroquine racemate. Each enantiomer was validated over the range of 1.5–30 µg/mL corresponding to 0.05–1% of the minor enantiomer based on the concentration of 3 mg/mL of the major enantiomer. The LOQ estimated at a signal-to-noise ratio of 10 was 0.05% (1.5 µg/mL) for the enantiomers and the LOD estimated at a signal-to-noise ratio of 3 was 0.02% (0.6 µg/mL). Correlation coefficients of at least 0.997 were observed indicating sufficient linearity in the investigated concentration range. The RSD values of intraday and interday precision at high and low concentrations were generally below 6%. A synthetic sample of (*R*)-(–)-chloroquine was subsequently analyzed using the validated method. An electropherogram of a sample containing 3 mg/mL (*R*)-(–)-chloroquine and 40 µg/mL carbamazepine as internal standard is shown in Fig. 16. A concentration of $0.24 \pm 0.01\%$ (mean \pm SD, $n = 3$) of the (*S*)-(+)-enantiomer could be detected as well as another impurity (peak X) at a migration time of about 13 min. A concentration of another impurity $0.41 \pm 0.02\%$ was estimated by peak area normalization. Peak X was not the synthetic precursor 4,7-dichloroquioline.

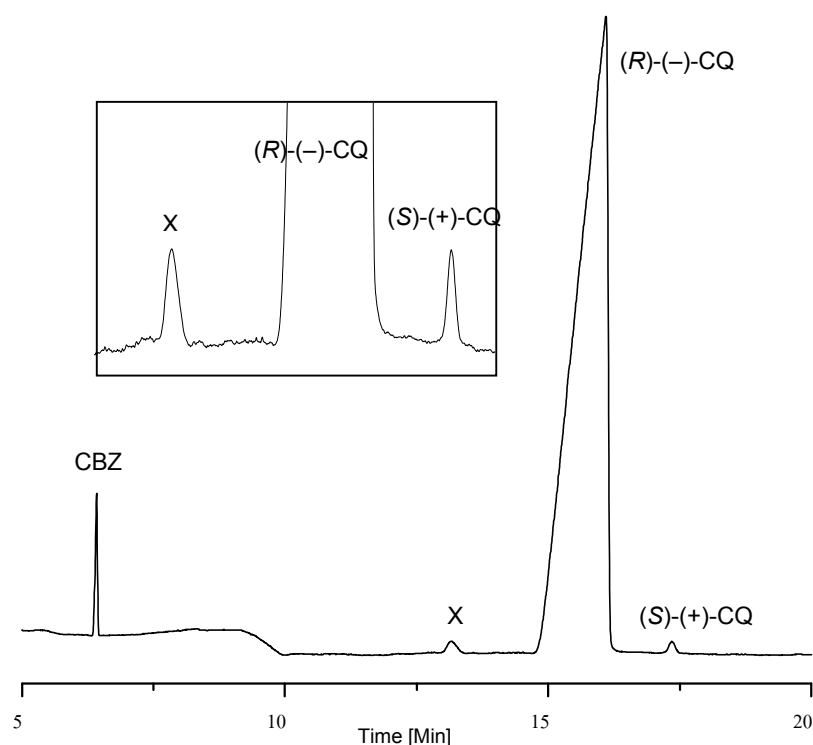


Figure 16. Electropherogram of a sample containing 3 mg/mL (*R*)-(-)-chloroquine ((*R*)-(-)-CQ) with about 0.3% (*S*)-(+)-chloroquine ((*S*)-(+)-CQ) as well as 40 µg/mL carbamazepine (CBZ, IS) using the optimized method. Experimental conditions: 50.2/40 cm, 50 µm id fused-silica capillary, 100 mM sodium phosphate buffer, pH 2.5, 30 mg/mL SBE(VII)-β-CD, -25 kV, 20°C, 225 nm.

3.4 Generic method development and optimization of the stereoselective CE assay for the determination of stereochemical purity of charged basic compounds and uncharged compounds (Manuscript I, II, III, IV and V)

With regard to the present studies the development and optimization of CE methods for the simultaneous separation of the major compounds and the corresponding minor chiral and achiral compounds were achieved by various actions such as the variation of the compositions in the background electrolyte, the buffer concentration and pH, type of CDs and modes of CE technique. Thus, the factors affecting the enantioseparation as well as the general suggestions for method development and optimization in order to separate the stereoisomers and related compounds based on the present studies as described previously are discussed as follows. Chiral compounds containing basic functional group especially primary and secondary amines are most applicable.

3.4.1 Type of CDs

Initial screening tests employing various types of CDs are necessary. Due to the data obtained from the five present studies the use of anionic CD derivatives, especially sulfated β-CD and SBE-β-CD, shows a clear advantage over native CDs or neutral and positively charged CD derivatives. Moreover, SBE-β-CD proved to be an effective chiral selector for both charged and uncharged compounds. Samples containing very different contents of the major compound and minor analytes should be used in order to evaluate the separation efficiency of the CD derivatives. For example, the peak deformation of the minor enantiomer, (*S*)-(+)-chloroquine, was observed upon injection of

a sample of 2 mg/mL (*R*)-(-)-chloroquine and the peak deformation could be resolved by using SBE(VII)- β -CD instead of sulfated β -CD.

3.4.2 CD concentration

The variation of CD concentrations is used as one of the strategies in order to improve the resolution and peak shape of investigated analytes. Generally, increasing the CD concentration results in a good enantioresolution, but increasing the CD concentration above the maximum value may result in an excess of electric currents and consequently a poor resolution due to Joule heating generation as well. Therefore, there is an optimum CD concentration for a particular compound at which chiral resolution reaches a maximum value. For example, in case of the CD-modified MEEKC system the resolution of the amphetamine enantiomers was improved by increasing the contents of sulfated β -CD from 4% to 5.5%, but a further increase in sulfated β -CD above 6% resulted in a comigration of some peaks as well as an unstable baseline. Moreover, an increase in the migration time with increasing concentrations of sulfated β -CD was observed. The effect of the CD concentration on the migration time depends on the charge of both, the chiral selector and the analyte as well as the polarity of the applied voltage. In addition, an increase in the CD concentration also affects the peak shape as shown in the case of the distorted peak of the minor component levoamphetamine could be improved by increasing the concentration of HDAS- β -CD.

3.4.3 A single CD system or a dual CD system?

In some case, it is preferable to combine two CDs in order to improve the enantioresolution of chiral compounds when using a single CD is not effective. For example, a dual CD system comprising SBE(V)- β -CD for the sufficient separation of uncharged compounds and sulfated β -CD for the effective resolution of the amphetamine enantiomers proved to be superior to the use of a single CD system.

3.4.4 Degree of substitution (DS)

It was noted that the degree of substitution of the CDs affects the resolution as well as peak shape of analytes. In the case of SBE- β -CD with a DS of 4, 5 and 7, the baseline separation of dexamphetamine and related compounds could be achieved by using SBE(V)- β -CD, whereas a distorted peak of the major compound was observed for SBE(IV)- β -CD and a decrease in peak resolution between the amphetamine enantiomers was observed for SBE(VII)- β -CD. Moreover, in case of chloroquine the separation of the enantiomers could be achieved using SBE(VII)- β -CD while SBE(V)- β -CD was insufficient. Thus, the enantioseparation of investigated compounds under the variation of DS of the selected CDs should be also evaluated.

3.4.5 Batches of CD

In case of CDs with randomly substitution patterns or qualities, e.g., sulfated β -CD, the qualities of substituents in each batch affect the performance of the analytical method. On the other hand,

batches of randomly substituted CD derivatives affect the reproducibility of the analytical methods. As demonstrated in the separation of levodopa and related substances, three batches of commercial sulfated β -CD from different suppliers resulted in a different resolution, especially the resolution between levodopa and Tyr as a critical pair.

3.4.6 Type of buffer and electrolyte

Based on the present studies sodium phosphate buffers seem to be most effective for the stereoselective CE assay of basic compounds. In the case of levodopa and related substances, it was noted that using a phosphate buffer prepared from orthophosphoric and sodium hydroxide buffer molarity as well as the concentration of sulfated β -CD could be reduced with comparable separation to the use of a phosphate buffer prepared from sodium dihydrogen phosphate. In addition, using Tris-phosphate buffers the observed electric currents are lower than using sodium phosphate buffer.

3.4.7 Buffer concentration

The buffer concentration affects peak shape as well as resolution of analytes. Theoretically, the adsorption of analytes to the capillary walls is reduced upon an increase in the buffer concentration while an increase in ionic strength can reduce the electromigration dispersion, therefore the separation and peak efficiencies are obtained [25, 51, 81]. In case of the analysis of dexamphetamine employing HDAS- β -CD, an increase in the buffer concentration resulted in sharp peaks of the later migrating levoamphetamine. Moreover, in case of the analysis of the chloroquine enantiomers peak shape as well as resolution of the enantiomers was improved upon increasing the buffer molarity from 50 mM to 100 mM. However, increasing the buffer concentration leads to an increase in electric currents as well. With regard to the data obtained from the present CE assays, the optimized methods frequently employ the phosphate buffer at concentrations between 50 and 100 mM.

3.4.8 Buffer pH

Buffer pH affects migration time as well as peak shape and resolution of the analytes. For example, in case of the analysis of dexamphetamine employing HDAS- β -CD an increase in the buffer pH from 2.5 to 3.5 and 6.5 led to shorter migration times but also poorer peak shape. As amphetamine is a basic compound that is protonated at low pH increasing the interaction to CDs, therefore the buffer pH can affect the migration time and the peak shape of analytes [30, 51]. However, the effect of the buffer pH is sometimes unpredictable. For example, regarding the analysis of levodopa reducing the buffer pH from 3.0 to 2.0 resulted in sharp peaks and short migration times. In case of a dual CD system a partial overlap of the isomers of phenylacetone oxime could be resolved by increasing the buffer pH from 2.5 to 3.0, but a longer migration time was observed. Furthermore, the buffer pH affects the performance of the assay. For example, in case of the analysis of dexamphetamine employing CD-modified MEEKC comigration and peak splitting were obtained using a BGE at pH 9.5 whereas a sufficient resolution and peak shape could be achieved using suppressed electroosmosis CD-modified MEEKC employing a BGE at pH 3.0. Based on the data

obtained from the present CE assays, the optimized methods frequently employ the phosphate buffer at pH between 2.0 and 3.0 for the separation of basic and neutral compounds.

3.4.9 Compositions in microemulsion and in the background electrolyte

In case an assay employing CD-MEEKC is selected, a variation of the composition of the microemulsion and the BGE can be used to optimize the selectivity of the method. Initially, the microemulsion consisting of 1.4 – 2% surfactant (e.g. SDS), 0.5 – 1% of an oil core (e.g. ethyl acetate, n-heptane) and 3 – 4% co-surfactant (e.g. 1-butanol, 2-propanol, 1-pentanol) with or without an organic modifier (e.g. 2-propanol, methanol, acetonitrile) should be evaluated. The addition of CDs in order to accomplish stereoisomer separation should be subsequently evaluated by screening of CDs and variation of CD concentrations. The buffer molarity and buffer pH used for the preparation of microemulsion should be evaluated as well. Moreover, it was observed that the addition of CD either before or after preparation of a microemulsion may affect the resolution of analytes.

3.4.10 Normal polarity or reversed polarity?

Modes of the applied voltage, normal polarity and reversed polarity, affect the mobility and consequently the separation of investigated compounds. The selection of normal or reversed polarity depends on the performance which is generally due to the properties of the analyte and the selected chiral selector. For example, in case of the analysis of dexamphetamine employing a dual CD system none of neutral compounds could be detected using the normal polarity of the applied voltage. In contrast, using the reversed polarity mode charged and neutral compounds could be detected simultaneously. In addition, using the reversed polarity mode the BGE containing a high concentration of CDs with electric currents below 100 μA is allowed, which may result in an improvement in the enantioseparation of analytes. For example, in case of the dual CD system electric currents of about 75 μA were observed when the optimized BGE containing the amount of SBE(V)- β -CD as high as 80 mg/mL and 25 mg/mL sulfated β -CD was employed. The superiority of reversed polarity of the applied voltage was also observed for the analysis of the chloroquine enantiomers employing SBE(VII)- β -CD as chiral selector. However, in case of the analysis of dexamphetamine employing HDAS- β -CD and the example of levodopa the separation could be achieved by using the normal polarity of the applied voltage.

3.4.11 Applied voltage

An increase in the applied voltage results in short analysis times. In some case the resolution is also improved when the applied voltage is increased. However, increasing the applied voltage can lead to excessive currents which result in Joule heating. With regard to the data obtained from the present CE assays, the optimized methods frequently employed the applied voltage at between +20 and +25 kV (normal polarity) or –10 to –25 kV (reversed polarity).

3.4.12 Length of the capillary

Based on the data obtained from the present CE assays, the optimized methods were frequently performed in a 50 μm id fused-silica capillary with an effective length between 30 and 50 cm. A decrease in an effective length of capillary generally results in a short analysis time, however the resolution may be reduced as well. In addition, an increase in electric currents was observed when the effective length was shortened.

3.4.13 Temperature

An increase in temperature of the capillary generally gives rise to the electric current generation and poor resolution. With regard to the data obtained from the present CE assays, the optimized methods are frequently operated at a temperature between 15 and 20°C.

3.4.14 Sample concentration and injected amount

An increase in the concentration of the major compound as well as an increase in the injection time can be used in order to enable the determination of impurities at the 0.1% level or below. However, peak deformation or peak tailing of the major compound as well as a partial overlap of the enantiomers can be also observed due to an increase in sample concentration as well as long injection times. Based on the data obtained from the present CE assays, a hydrodynamic injection at a pressure of 0.5 psi for 4 to 8 sec is usually employed in the optimized method.

3.4.15 Preconditioning of the capillary

Preconditioning of the capillary before sample introduction may affect the stability of the baseline as well as the resolution and the peak shape of the analytes. A 0.1 M sodium hydroxide solution is commonly used for the preconditioning process followed by water and either the BGE or the buffer (no CD). In case of CD-modified MEEKC methanol is usually included in the preconditioning process. Interestingly, regarding the analysis of the chloroquine enantiomers the use of the BGE in preconditioning led to currents above 100 μA as well as a poor resolution. In contrast, using the buffer without CD in preconditioning resulted in a good resolution of the chloroquine enantiomers and currents between 80 and 90 μA were observed.

3.4.16 Schematic guidance for method development and optimization

Based on the data obtained from the present stereoselective CE assays for the simultaneous determination of various phenylethylamine derivatives as well as related charged and neutral compounds, different strategies used for achieving chiral and non-chiral separation are purposed. A generic guidance for development and optimization of a CE method for the determination of stereochemical purity and related impurities is shown in Fig. 17 as a schematic flow chart. The guidance in Fig. 17 is adapted from the guidance published by Sokoließ and Köller [82]. Compounds containing basic functional group, especially primary and secondary amines, as well as neutral functionalities are most applicable for this guidance. In addition, in case of the chloroquine enantiomers the method development and optimization was conducted according to

this guidance, thus a short time was spent in order to accomplish the enantioseparation of (\pm)-chloroquine.

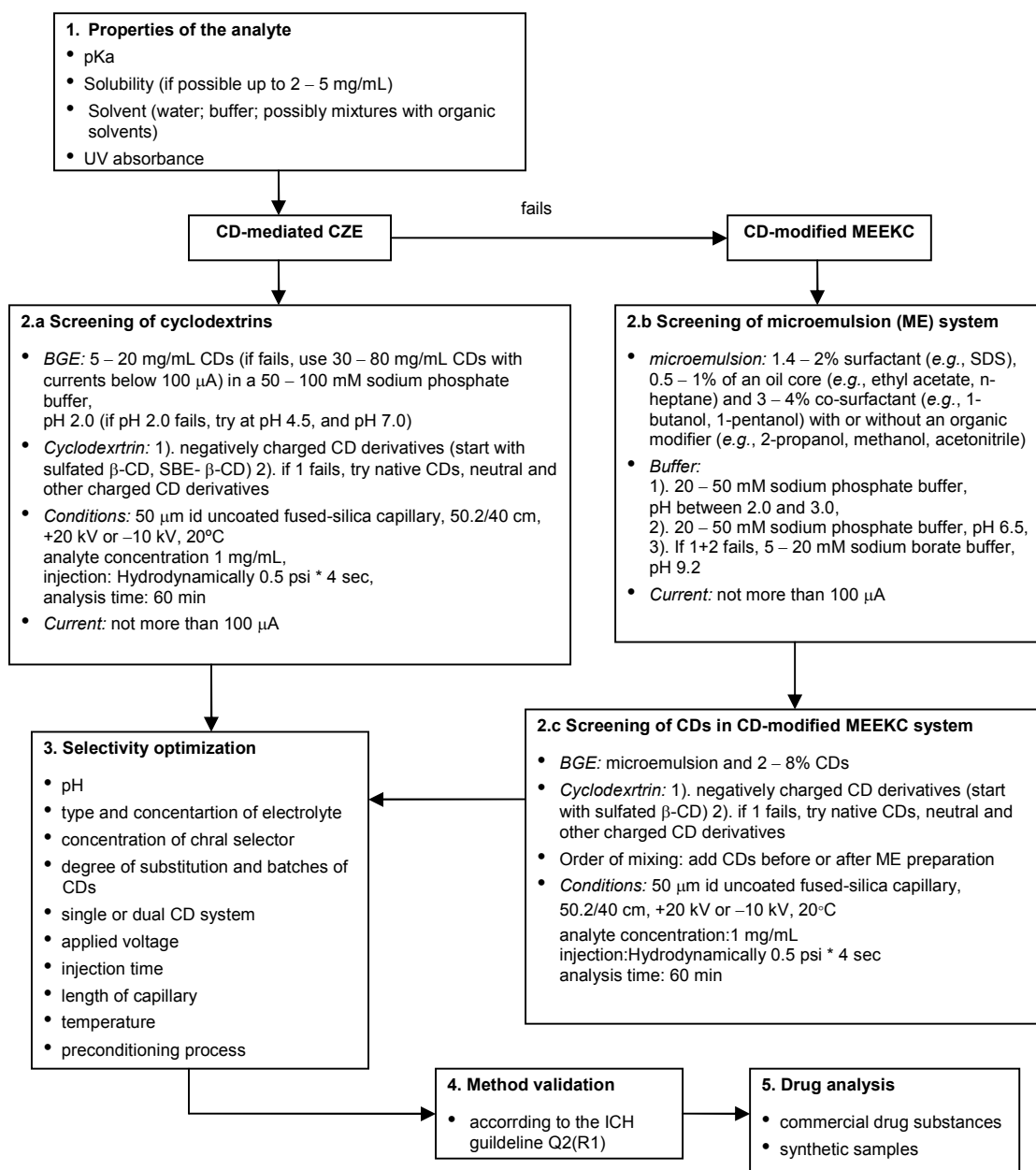


Figure 17. Guidance on development and optimization of CD-mediated CZE and CD-modified MEEKC methods for the simultaneous determination of stereochemical purity and related compounds of basic drugs (adapted from Ref. [82]).

Summary

The stereochemical configuration of drugs plays an important role for their biological activities due to the stereoselectivity of enzymes, receptors and other molecular targets. This is also reflected in the fact that single enantiomer drugs have become a significant proportion in worldwide drug sales. Generally, the safety and efficacy of a drug does not only depend on the active ingredient itself but also on impurities. In case of chiral drugs the distomer has to be considered as well. Therefore, the control of the enantiomeric purity of a drug substance as well as the related substances is an important concern of pharmaceutical companies and regulatory authorities. Traditionally, polarimetry is used for the determination of the enantiomeric purity while HPLC is applied to the determination of related substances. In recent years CE has been acknowledged as a powerful technique allowing the analysis of closely related structures as well as stereoisomers. Therefore, the aim of the thesis was the development and validation of stereoselective CE assays allowing the simultaneous determination of related substances as well as enantiomeric purity of chiral drugs.

The single enantiomer drugs dexamphetamine and levodopa as well as the racemic drug chloroquine were selected as target analytes. CDs were employed as chiral selectors because they are most widely used in CE enantioseparations.

Three different CE assays were developed and validated for the analysis of dexamphetamine including a single CD system, a dual CD system and a CD-modified MEEKC method. The initially developed single CD method allowed the determination of the charged potential impurities 1*R*,2*S*-(-)-norephedrine and 1*S*,2*S*-(+)-norpseudoephedrine besides the analysis of the enantiomeric purity. Heptakis-(2,3-di-O-acetyl-6-O-sulfo)- β -CD (HDAS- β -CD) was chosen as chiral selector based on a screening of neutral and charged CD derivatives. The assay was subsequently validated employing 1*R*,2*S*-(-)-ephedrine as internal standard. Analysis of commercial samples dexamphetamine sulfate drug substance revealed the presence of 2.8 - 4.0% of the levorotatory enantiomer but none of the charged impurities could be detected. Apparently, dexamphetamine is not synthesized from the chiral precursors.

Therefore, a second assay allowing also the determination of neutral synthetic precursors such as phenylacetone and phenylacetone oxime was developed and validated. Following a screening of neutral and negatively charged CDs, sulfated β -CD and sulfobutylether β -CD with degree of substitution of 5 (SBE(V)- β -CD) were selected due to their abilities for the separation of both charged and neutral compounds. In the dual CD system sulfated β -CD effectively separated the amphetamine enantiomers while SBE(V)- β -CD resolved the related charged and uncharged substances. The present assay was able to separate the geometrical isomers *E*- and *Z*-isomers of phenylacetone oxime. As observed for the single CD assay neither 1*R*,2*S*-(-)-norephedrine nor 1*S*,2*S*-(+)-norpseudoephedrine could be detected. In contrast, phenylacetone as well as the *E*- and *Z*-isomers of phenylacetone oxime could be detected indicating the synthetic origin of the samples. The distomer levoamphetamine was detected as well.

Finally, a CD-modified MEEKC assay was developed and validated as an alternative method. The background electrolyte consisted of the microemulsion containing sodium dodecyl sulfate (SDS), ethyl acetate, 1-butanol, 2-propanol and a 50 mM sodium phosphate buffer. The chiral selector was

sulfated β -CD. The effects of the composition of the microemulsion, the CD concentration, the buffer concentration, the buffer pH and the applied voltage on the resolution of all analytes were investigated. Interestingly, the addition of sulfated β -CD to the BGE either before or after the preparation of the microemulsion slightly affected the resolution of some analytes. The method was validated using carbamazepine as internal standard. Subsequently, commercial samples of dexamphetamine sulfate were analyzed. Only levoamphetamine could be detected due to the fact that the LOD of the MEEKC method was higher compared to the single or dual CD assays.

Comparing the three CE assays, the dual CD method was superior due to its ability to allow the analysis of charged and uncharged compounds as well as due to the low LOD. A clear disadvantage is the use of expensive SBE(V)- β -CD in the assay.

The stereoselective CE assay developed for levodopa used sulfated β -CD at a concentration of 6 mg/mL in a 0.1 M sodium phosphate buffer, pH 2.0. The method was validated using *L*-phenylalanine as internal standard. Three samples of commercial levodopa drug substance were subsequently analyzed. Neither 6-hydroxy-DOPA nor 3-methoxy-*L*-tyrosine could be detected in the samples, whereas *L*-tyrosine was detected in all samples at concentrations below the LOQ. *D*-DOPA was found in two samples.

Finally, a stereoselective CE assay was developed and validated in order to determine the enantiomeric purity of synthetic *R*-(-)-chloroquine. The method was initially developed and optimized using sulfated β -CD as a chiral selector. However, upon an injection of a sample containing 2 mg/mL *R*-(-)-chloroquine peak deformation of the minor component *S*-(+)-chloroquine was observed. Peak distortion could be avoided using SBE(VII)- β -CD as chiral selector, which also allowed to increase the sample concentration to 3 mg/mL. Carbamazepine was used as internal standard. Upon analyzing a synthetic sample of *R*-(-)-chloroquine, *S*-(+)-chloroquine was found at a concentration of $0.24\% \pm 0.01\%$ (means \pm SD). Moreover, an unknown impurity with a shorter migration time was also detected at the 0.4% level calculated from normalized peak area.

In conclusion, the methods developed in the thesis proved the applicability of CE for the simultaneous determination of the enantiomeric purity and related substances of the drugs dexamphetamine, levodopa and chloroquine. The assays had acceptable peak efficiency, sensitivity and precision. Moreover, they could be used to analyze commercial samples. Thus, the method could be considered valuable alternatives to the current methods used by the European Pharmacopoeia or the United States Pharmacopeia.

Zusammenfassung

Die stereochemische Konfiguration von Arzneistoffen beeinflusst ihre biologische Aktivität aufgrund der stereoselektiven Interaktion mit Enzymen, Rezeptoren und anderen molekularen Targets. Dies zeigt sich auch in der Tatsache, dass enantiomerenreine Arzneistoffe einen signifikanten Anteil an den weltweiten Pharmaumsätzen besitzen. Die Sicherheit und Effizienz von Arzneistoffen wird nicht nur vom Wirkstoff sondern auch von Verunreinigungen bestimmt. Im Fall enantiomerenreiner Arzneistoffe muss das Diastomer auch als Verunreinigung angesehen werden. Daher ist die Bestimmung der Enantiomerenreinheit für Pharmaunternehmen und Zulassungsbehörden von großer Bedeutung. Traditionellerweise wird die Enantiomerenreinheit mittels Polarimetrie bestimmt, während verwandte Substanzen mittels HPLC begrenzt werden. In den letzten Jahren hat sich die Kapillarelektrophorese (CE) als eine Analysentechnik zur Bestimmung nahe verwandter Substanzen einschließlich Stereoisomeren entwickelt. Daher war das Ziel der Forschungsarbeiten die Entwicklung und Validierung stereoselektiver CE Verfahren, die die simultane Bestimmung verwandter Substanzen und der Enantiomerenreinheit chiraler Arzneistoffe erlauben.

Als Substanzen wurden die chiralen Wirkstoffe Dexamphetamin und Levodopa ausgewählt sowie der racemische Arzneistoff Chloroquin. Als chirale Selektoren wurden Cyclodextrine (CDs) als die für chirale Trennungen am häufigsten eingesetzten Selektoren eingesetzt.

Für Dexamphetamin wurden 3 CE Assays entwickelt und validiert – ein Verfahren unter Verwendung eines CDs, ein Verfahren mit dualem CD System sowie ein Verfahren mittels CD-modifizierter Mikroemulsion elektrokinetischer Chromatographie (MEEKC). Das zuerst entwickelte Verfahren mit einem CD erlaubt die Bestimmung der geladenen Verunreinigungen 1*R*,2*S*-(–)-Norephedrin und 1*S*,2*S*-(+)-Norpseudoephedrin sowie die Bestimmung der Enantiomerenreinheit. Als chiraler Selektor wurden Heptakis-(2,3-Di-O-acetyl-6-O-sulfo)- β -CD (HDAS- β -CD) eingesetzt. Der Assay wurde mit Ephedrin als internem Standard validiert. In kommerziellen Proben wurden 2.8 bis 4.0% des linksdrehenden Amphetamin-Stereoisomers gefunden. Keine der geladenen Verunreinigungen konnte nachgewiesen werden, so dass von einem anderen Syntheseweg als über die Naturstoffe ausgegangen werden muss.

Daher wurde ein zweiter Assay entwickelt und validiert, der auch die Bestimmung neutraler Synthesestufen Phenylacetone und Phenylacetonoxyms erlaubt. Nach einem Screening wurden sulfatiertes β -CD und Sulfobutylether- β -CD mit einem Substitutionsgrad von 5 (SBE(V)- β -CD) aufgrund ihrer Trenneigenschaften ausgewählt. In dem dualen CD-System erfolgt die Enantiomerentrennung des Amphetamins in erster Linie durch das sulfatierte β -CD, während die Trennung der geladenen und ungeladenen verwandten Substanzen durch SBE(V)- β -CD erfolgt. In dem Assay werden auch die E- und Z-Isomere des Phenylacetonoxyms getrennt. Wie im ersten Assay konnten die geladenen potentiellen Verunreinigungen nicht nachgewiesen werden, aber es wurden Peaks des Phenylacetons und der Oxim-Stereoisomere gefunden. Dies belegt die Synthese der Substanz. Levoamphetamin wurde ebenfalls gefunden.

Zusätzlich wurde eine CD-modifizierte MEEKC-Methode als alternatives Verfahren aufgebaut. Der Hintergrundelektrolyt bestand aus einer Mikroemulsion aus Natriumdodecylsulfat (SDS), Ethylacetat, 1-Butanol, 2-Propanol und 50 mM Natriumphosphat-Puffer. Der chirale Selektor war

sulfatiertes β -CD. Die Zusammensetzung der Mikroemulsion, die CD Konzentration, die Pufferzusammensetzung, der Puffer pH sowie die angelegte Spannung wurden optimiert. Interessanterweise hatte der Zeitpunkt der Zugabe des CDs (vor oder nach Herstellung der Mikroemulsion) einen Einfluss auf die Auflösung einiger Peaks. Nach der Validierung unter Verwendung von Carbamazepin als interner Standard wurden Substanzproben vermessen. Aufgrund der geringeren Nachweisgrenze verglichen mit dem dualen CD-Assay konnten außer Levoamphetamin keine anderen Verunreinigungen nachgewiesen werden.

Im Vergleich der 3 Methoden erwies sich der Assay mit dualen CD-System als universeller und sensitiver zur Bestimmung der untersuchten potentiellen Verunreinigungen. Allerdings ist SBE(V)- β -CD teuer.

Bei der stereoselektiven Methode für Levodopa wurde wiederum sulfatiertes β -CD als chiraler Selektor in einer Konzentration von 6 mg/mL in 0.1 M Natriumphosphatpuffer mit pH 2.0 eingesetzt. L-Phenylalanin diente als interner Standard. Bei der Untersuchung von 3 Substanzproben konnte von den im Europäischen Arzneibuch aufgeführten Nebenprodukten weder 6-Hydroxy-DOPA noch 3-Methoxy-L-tyrosin nachgewiesen werden, während L-Tyrosin in allen Proben unterhalb der Bestimmungsgrenze detektiert wurde. 2 Proben enthielten auch geringe Mengen an D-DOPA.

Zur Bestimmung der Enantiomerenreinheit von synthetischem R-(–)-Chloroquin wurde ursprünglich ein Verfahren unter Verwendung von sulfatiertem β -CD untersucht. Nach Injektion einer Probe vom R-(–)-Chloroquin mit einer Konzentration von 2 mg/mL wurde die Deformation des Peaks des in geringer Konzentration vorhandenen S-(+)-Chloroquin beobachtet. Daher wurde sulfatiertes β -CD durch SBE(VII)- β -CD ersetzt, was gleichzeitig die Erhöhung der Konzentration der Probe auf 3 mg/mL erlaubte. Carbamazepin diente als interner Standard. Der Anteil an S-(+)-Chloroquin in einer Probe R-(–)-Chloroquin betrug $0.24\% \pm 0.01\%$ (Mittelwert \pm SD). Außerdem wurde eine zusätzliche unbekannte Substanz im Bereich von 0.4% nach Normalisierung gefunden.

Zusammenfassend kann festgestellt werden, dass die im Rahmen der Forschungsarbeiten für Dexamphetamin, Levodopa und Chloroquin entwickelten CE Methoden zur simultanen Bestimmung der Enantiomerenreinheit und der verwandten Substanzen geeignet sind. Die Verfahren besitzen ausreichende Effizienz, Sensitivität und Präzision. Kommerzielle Substanzproben konnten untersucht werden. Daher können die neuen Verfahren Alternativen zu den derzeit im Europäischen Arzneibuch und der United States Pharmacopoe vorgeschriebenen Tests darstellen.

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List of publications

- **Profiling of levoamphetamine and related substances in dexamphetamine sulfate by capillary electrophoresis**
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Poster presentations

- *“Determination of clarithromycin in human serum by high-performance liquid chromatography with electrochemical detection”*
S. Kumlai, K. Ingkaninan, The Forth Indochina Conference on Pharmaceutical Sciences, Ho Chi Minh City, Vietnam
- *“Impurity Assays of Drugs Using Capillary Electrophoresis”*
B. Sungthong, S. Wongwan, G. K. E. Scriba, Symposium Analytical Chemistry of the Association of German Chemists, Hohenroda, Germany, January 2008

- *“Development and Validation of Capillary Electrophoresis Assay for the Determination of the Stereochemical Purity of Dexamphetamine”*

N. G. Kokiashvili, M. Hammitzsch-Wiedemann, S. Wongwan, G. K.E. Scriba, 27th

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- *“Determination of the Enantiomeric Purity of Dexamphetamine by Capillary Electrophoresis”*

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Selbstständigkeitserklärung

Entsprechend der geltenden, mir bekannten Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Dissertation eigenständig angefertigt und alle von mir benutzten Hilfsmittel und Quellen angegeben habe. Personen, die mich bei der Auswahl und Auswertung des Materials sowie bei der Fertigstellung der Manuskripte unterstützt haben, sind am Beginn eines jeden Kapitels genannt. Es wurde weder die Hilfe eines Promotionsberaters in Anspruch genommen, noch haben Dritte für Arbeiten, welche im Zusammenhang mit dem Inhalt der vorliegenden Dissertation stehen, geldwerte Leistungen erhalten. Die vorgelegte Dissertation wurde außerdem weder als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung noch als Dissertation an einer anderen Hochschule eingereicht.

Sudaporn Wongwan

Jena, den 08.12.2010